NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETEAL AND SKIN DISEASES

ANNUAL REPORTS

INTRAMURAL RESEARCH PROGRAMS

OCTOBER 1, 1990 TO SEPTEMBER 30, 1991

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PREFACE

The National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) is responsible for research on a broad range of diseases and conditions that at one time or another involve almost every family in the Nation. Most of these diseases are long lasting. Many are severely disabling. They generate a tremendous burden of human suffering and economic loss, both to the individuals affected and to society.

The Intramural Research Program (IRP) of the Institute consists of four major investigational entities: the Arthritis and Rheumatism Branch (ARB), the Laboratory of Physical Biology (LPB), the Laboratory of Structural Biology Research (LSBR), and the Laboratory of Skin Biology (LSB). Dr. Henry Metzger, an internationally renowned immunologist, is the Director of the IRP. Dr. John H. Klippel, a highly talented clinical investigator of rheumatic diseases, is the IRP's Clinical Director.

The Arthritis and Rheumatism Branch was established over 30 years ago as a major intramural component of what was then the National Institute of Arthritis and Metabolic Diseases. It has been one of the outstanding centers for laboratory and clinical research and research training in rheumatology, basic immunology, and related areas. Research in the Branch is directed by five senior investigators and covers a broad spectrum of disciplines: immunology, molecular biology, genetics, biochemistry, physiology, virology, pharmacology, cell biology, pathology, and clinical investigations. In addition to collaborations within the Branch, there are many cooperative projects with other groups at the National Institutes of Health. The Branch is organized into three sections.

In the Section on Cellular Immunology, genetic and cellular abnormalities that underlie human and murine autoimmune diseases are explored. Emphasis is placed on research on systemic lupus erythematosus. Studies cover the range of fundamental investigations on the nature and development of B lymphocyte repertoires to the development and evaluation of experimental therapies. Also, researchers are trying to identify the gene for Familial Mediterranean Fever, an autosomal recessive disease that causes regular, periodic bouts of fever and rheumatic symptoms. Location of the gene and future studies on the protein for which it codes should drive further investigations of the biochemistry that underlies this mysterious disease.

In the Section on Connective Tissue Diseases, researchers explore the etiology and pathogenesis of autoimmune diseases. In particular, an excellent rat model of rheumatoid arthritis is being studied with recent emphasis placed on the effect of the hypothalamic-adrenocortical axis on the course of the disease. Polymyositis in humans and mice is also being thoroughly investigated, with special emphasis on the use of highly sensitive genetic probes to look for possible viral etiologic agents.

The NIAMS is proud to have supported recent work by the Section on Chemical Immunology, which discovered the complete structure of the immunoglobulin E receptor (IgE) by cloning the IgE genes and determining the resultant proteins. This IgE receptor is an essential agent for developing allergic responses. Its activity on mast cells serves as a model system to address basic questions on the mechanism by which cells bearing antibody receptors are activated. Structure-function relationships are currently being explored by molecular genetic approaches.

Clinical investigations at the ARB include studies on patients with systemic lupus erythematosus, rheumatoid arthritis, and polymyositis. Through a unique 20-year followup study, ARB investigators have found that fewer lupus patients with kidney disease (a common complication) progress to kidney failure when given intravenous cyclophosphamide rather than two other commonly used treatment protocols. This finding has been widely adopted by physicians treating lupus patients. In addition, recent findings from a unique collection of patient tissues have enabled the researchers within the Section on Connective Tissue Diseases to learn that human polymyositis can be divided into subsets based on a patient's antibody production. This classification may help physicians target treatments more effectively.

A second major component of the IRP is the Laboratory of Physical Biology (LPB), the underlying scientific disciplines of which are biophysics and structural biology. The Laboratory studies biological problems using a broad range of physical techniques. They include X-ray diffraction, high resolution mechanical recording electron microscopy, image processing, surface film techniques, and radiation inactivation analysis. The Laboratory consists of two sections.

In the Section on Muscle Biophysics, researchers study the mechanisms of muscle regulation and contraction studied by methods that assess its structural and functional aspects. The combination of data from structure-revealing techniques (X-ray diffraction, electron microscopy) and function-revealing techniques (force and displacement recording, radiation inactivation analysis) lends this Section a unique capability to optimally address specific questions in muscle physiology.

In the Section on Macromolecular Biophysics, the structure and physical states of lipids in cell membranes are explored using model systems of lipid dispersions in water. The instrument to gather relevant data, a quasi-adiabatic scanning microcalorimeter, is one of a kind. Researchers in this Section study the physicochemical relationships between diseased states in tissues and membrane bilayer instability. In other work, the effect of ionizing radiation on macromolecules is used to determine the in vivo size of the functional units for different biological activities (hormone receptors, enzyme complexes, and regulatory units).

The Laboratory of Structural Biology Research (LSBR) is a third major component of the IRP. The LSBR investigates mechanisms of macromolecular assembly and structure-function relationships at the supramolecular level by high resolution microscopy in conjunction with digital image processing and by cryo-electron microscopy. Computer modeling is also used to integrate data from other biochemical and biophysical sources. Systems of interest include the structure and assembly of viruses, intermediate filaments including keratin filaments, clathrin and coated membranes, and other fibrous polymers and oligomeric protein complexes. Recent studies by the LSBR of the DNA packaging in herpes virus particles have revealed a close parallel between viral assembly in a major family of animal viruses (herpes) and a well-studied family of bacterial viruses (bacteriophages). Existing knowledge of the bacteriophage system now suggests specific research avenues that can be explored to understand the herpesvirus system.

The final component of the IRP is the Laboratory of Skin Biology, which is undergoing a rapid growth phase during its first year and a half of existence. The Laboratory now has a strong section in molecular biology and

structure that has recently unveiled the assembly of keratin intermediate filament chains. These filaments are expressed in terminally differentiated epidermal cells, those that are turning into the flattened, outermost skin cells that provide a barrier function. A new section on human genetics will shortly join the LSB and focus on the genetics underlying inherited keratinizing disorders, including the ichthyoses. Recruitment of clinical dermatologists for a third integrated section will enable the LSB to perform a complete range of investigations from laboratory to clinic.

In its extramural research programs, the Institute supports a spectrum of basic, clinical, and epidemiologic research and research training in the important fields of rheumatology, orthopaedics, bone biology and bone diseases, muscle biology, and dermatology.

Major avenues of arthritis research supported through the Arthritis Program include humoral and cellular immune abnormalities in the pathogenesis of rheumatic disease; mechanisms by which mediators of inflammation, such as the prostaglandins and leukotrienes, promote and regulate inflammatory reactions in arthritis; immunogenetic factors underlying susceptibility to arthritis; ultrastructure and pathophysiology of collagens, proteoglycans, and other components of connective tissue; and metabolic derangements underlying osteoarthritis and gout/pseudogout.

The Musculoskeletal Diseases Program supports studies of joint disorders of the musculosketal system and therapies such as joint replacement and bone and cartilage transplantation to treat these disorders. In addition, sports

medicine and musculoskeletal fitness and low back pain are areas of special research emphasis.

The Bone Biology and Bone Diseases Program supports basic and clinical studies of bone diseases, including osteoporosis, a disease affecting the Nation's growing senior citizen population. Other diseases studied include heritable disorders such as osteogenesis imperfecta (the brittle bone disease) and Paget's disease (the disfiguring disorder that causes irregular bone growth).

Researchers supported by the Muscle Biology Program are investigating normal muscle development and function, as well as the pathophysiology of muscle disorders and diseases. Research can be placed into five broad categories: (1) structure and function of muscle, (2) development and regeneration of muscle, (3) muscle energetics and metabolism, (4) mechanisms of excitation-contraction coupling, and (5) muscle disease and disorders.

Investigations supported through the Skin Diseases Program are under way on a wide range of diverse problems, including psoriasis, acne, vitiligo, atopic dermatitis, and fatal bullous diseases. Employing new laboratory techniques, scientists are attempting to clarify the roles of metabolic abnormalities, phototoxicity, and immunopathology in the etiology of these cutaneous disorders.

In sum, research activities through the Intramural and Extramural programs have led to major findings that significantly increase the existing knowledge on the many disorders covered by the NIAMS mandate. This knowledge base allows further important investigations on the treatment and, eventually, the development of cures for these diseases. We take great pride in the

scientific achievements of our intramural scientists and in their high reputation within the scientific community.

Lawrence E. Shulman, M.D., Ph.D.
Director
National Institute of Arthritis and
Musculoskeletal and Skin Diseases

PROJECT NUMBERS
NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

ACTIVE PROJECTS

Z01 AR 27000-29 LPB

Z01 AR 27001-17 LPB

Z01 AR 27003-32 LPB

Z01 AR 27004-22 LPB

Z01 AR 27005-09 LPB

Z01 AR 27012-07 LPB

Z01 AR 27002-13 LSBR

Z01 AR 41020-24 ARB

Z01 AR 41023-17 ARB

Z01 AR 41025-20 ARB

Z01 AR 41040-19 ARB

Z01 AR 41048-12 ARB

Z01 AR 41066-09 ARB

Z01 AR 41074-04 ARB

Z01 AR 41076-04 ARB

Z01 AR 41080-03 ARB

Z01 AR 41083-02 ARB

Z01 AR 41088-01 ARB

Z01 AR 41084-02 LSB

Z01 AR 41085-02 LSB

Z01 AR 41086-02 LSB

Z01 AR 41087-02 LSB

INACTIVE PROJECTS

Z01 AR 41079-02 ARB
(Transferred to NIMH,
Z01 MH 02585-01 CNE)

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Muscle Contraction

The discovery of the weakly-binding actomyosin crossbridge in rabbit muscle fibers at low temperature and low ionic strength naturally led to questions concerning the physiological role of such crossbridges. The fact that the structure of the weakly-binding bridge differs from that of the strongly-binding bridge raised the possibility that force is generated by a transition between these two states. Many of the studies carried out this past year were concerned with this question.

Myosin head rotation

Previous equatorial X-ray diffraction studies have shown that a myosin head covalently crosslinked to actin in a rigor muscle becomes rotatable upon addition of ligands like ATP, ATPγS (a nonhydrolyzable analog of ATP) or pyrophosphate. To know how this rotation of myosin head is related to the mechanism of muscle contraction, a reverse-type experiment was carried out: a crosslinked muscle was brought into rigor by the removal of ATPγS from the bathing solution and the changes in force, stiffness and equatorial diffraction pattern were observed. Upon removal of ATPγS the crosslinked muscle produced force in proportion to the level of preapplied stretch (up to 1.5% muscle length), but no force was produced if the muscle had not been prestretched. At any prestretch level, the muscle stiffness nearly doubled and the equatorial pattern was fully converted from relaxed- to rigor-type patterns. When the same muscle was activated by calcium in an ATP containing solution, it produced much larger force, independent of the prestretch level, while its stiffness and equatorial pattern were intermediate between those under relaxed and rigor conditions. These results suggest that (1) myosin ligands confer rotatability to a myosin head by increasing its elasticity and (2) a rigor head and a head with a ligand have the same equilibrium orientation. In addition, they imply that physiological force is not produced by a simple transition between weakly-binding and strongly-binding crossbridges. Presumably there is another as yet uncharacterized force-generating mechanism in the muscle fiber. (Iwamoto, Podolsky)

Caldesmon inhibition of weakly-binding crossbridges

Last year caldesmon, a regulatory protein in smooth muscle, was used to inhibit the formation of weakly-binding crossbridges. Inhibition of crossbridge formation in the weak binding states caused inhibition of active force. Therefore, the weak binding states are essential precursors to force generation. However, the conditions of the experiments were at low temperature and low ionic strength to facilitate the detection of the weak binding crossbridges. Preliminary data have now been obtained at near physiological conditions: at

temperature as high as 20° C and ionic strength as high as 170 mM. X-ray diffraction data show that the actomyosin binding is decreased as concentration of caldesmon is increased; mechanically the fiber resting stiffness is reduced by caldesmon and more importantly, active force is inhibited. The extent of inhibition in active force level is very similar to that found at lower temperature and lower ionic strength. The results indicate that first, crossbridges do form to a significant extent in the weak binding states at near physiological conditions; secondly, inhibition of crossbridge attachment in the weak binding states is sufficient to inhibit active force. These results are evidence that attached weak binding crossbridges are essential for force generation in skeletal muscle. (Yu, Brenner)

Interaction of strongly-binding crossbridges with actin

The myosin molecule has two subfragment-1 heads that can bind to actin. With the weakly-binding crossbridge interaction these two heads appear to function independently. For the strongly-binding interaction, this is no longer the case. In a model of the strongly-binding interaction put forward by Schoenberg, the binding of one crossbridge head severely restricts the allowable sites for the binding of the second head. This has a very big influence on the behavior of the crossbridge, particularly its ability to sustain force when strained. This predicted influence can be examined by using ionic strength to change the strength of the binding of crossbridge heads. It was found that increasing ionic strength from moderate values ($\mu \sim 100$ mM) to high values ($\mu \sim 200$ mM) has three effects experimentally. It causes a large decrease in the half time for the force decay after a small stretch, it causes a significant decrease in the sigmoidicity of the nucleotide analogue concentration dependence of the "apparent rate constant" of force decay after a small stretch, and it causes a large decrease in the range of rate constants necessary to describe the multiexponential force decay. It causes the last of these by causing a much larger increase in the slowest rate constants of the decay than in the fastest rate constants. The results offer strong support for the proposed model of the actin - strongly-binding crossbridge interaction. (Schoenberg)

Weakly-binding crossbridges and temperature

In order to reduce the likelihood of a relaxed fiber becoming active, the initial experiments of weakly-binding myosin·ATP crossbridges were done at 5°C. It has now been shown in mechanical experiments that myosin·ATP crossbridges are observable at 20°C. Significantly, the Q_{10} temperature coefficient for crossbridge binding suggests that the weakly-binding myosin·ATP crossbridges are likely also to be found at 38°C, the body temperature of the living rabbit. (Barnett, Schoenberg)

Radial crossbridge force

Two parameters of the elastic behavior of attached crossbridges in the radial direction perpendicular to the fiber axis were examined. The equilibrium point where the radial force is zero was shown to be a function of the state of the crossbridge. The other parameter, i.e. stiffness per crossbridge, is very likely also a function of the state of the crossbridge, although the evidence is not as direct. The difference in radial elasticity most likely reflects structural differences of the attached crossbridges. This approach of determining radial elasticity should prove to be an effective technique as a first screening for

structural differences in attached crossbridges, since this approach does not depend on the number of crossbridges attached to actin, a factor which frequently encumbers interpretation of other structural studies. (Xu, Yu)

Resting tension in fibers from various muscles

Single fibers from human biceps or quadriceps muscles have up to five times lower resting tension upon passive stretch than that produced by stretching rabbit psoas muscle fibers. Since proximal limb muscles were used in the human muscle study, limb muscles of the rabbit were tested to see if they showed low levels of resting tension similar to those found in the human muscle fibers. Rabbit soleus muscle fibers turned out to exhibit very low levels of resting tension, similar to that previously found in the human fibers.

At sarcomere lengths were the slope of the resting tension-sarcomere length relation is low, electron microscopy of skinned fibers indicated that thick filaments move from the center to the side of the sarcomere during prolonged activation. As sarcomeres are stretched and the resting tension-sarcomere length relation becomes steeper, this movement is decreased. These results are consistent with a model in which elastic titin filaments link each end of the thick filament to the nearest Z-disc, produce resting tension upon stretch of the sarcomere, and provide a force which tends to position the thick filaments at the center of the sarcomere. The sarcomere length range over which thick filament movement decreases is higher in soleus than in psoas fibers, paralleling the different lengths at which the slope of the resting tension-sarcomere length relation increase. These results indicate that the large differences in resting tension between single psoas and soleus fibers are due to different tensions exerted by the elastic elements linking the end of each thick filament to the nearest Z-disc, i.e. the titin filaments.

Using a sensitive, quantitative electrophoretic technique, the individual rabbit soleus muscle cells were shown to contain the same concentrations of major myofibrillar proteins as the rabbit psoas fibers. The five fold difference in resting tension observed is therefore not due to different levels of elastic, tension bearing proteins. These results, along with a small difference in the electrophoretic mobility of titin between rabbit psoas and soleus fibers, suggest instead that mammalian muscle cells use at least two titin isoforms with different elastic properties to produce variations in resting tension. (Horowits)

Regulation of smooth muscle

During the onset of contraction in smooth muscle, there is a reasonable correlation between myosin light chain phosphorylation and force development. Ca^{2+}-calmodulin dependent activation of myosin light chain kinase appears to lead to phosphorylation of the 20 kDalton myosin light chain which stimulates the myosin crossbridges to produce force. A sensitive radio-assay was developed and used to correlate myosin light chain phosphorylation and force production during atrial natriuretic factor (ANF) induced relaxation of tracheal smooth muscle. Surprisingly, a large discrepancy between force and myosin light chain reduction in myosin light chain phosphorylation was found. When ANF relaxes a KCl-induced contraction, a 50% reduction in myosin light chain phosphorylation is accompanied by only a 30% reduction in force. When ANF relaxes a 10^{-7} M acetylcholine contraction, there is no detectable change in myosin light chain phosphorylation

but a 50% decrease in force. There is no explanation for these findings at present, especially since it was found that ANF applied to a relaxed fiber produces no change in either force or myosin light chain phosphorylation. (Qian, Schoenberg)

Cell Membranes

Aqueous suspensions of phospholipids, either synthetic or natural mixtures from cell membranes, have been used to study how membrane bilayers assemble. The studies are based on a theory which describes membrane bilayer assembly as a physicochemical process that occurs only at a critical point, the physiological temperature of the cell. The optimum conditions for membrane bilayer stability, a prerequisite for normal cellular function, occur at the critical point; membrane lipid composition and the critical temperature are interdependent. When either lipid composition or temperature deviate from the required critical conditions, the membrane bilayer becomes unstable and its structure degenerates.

Calorimetry of critical bilayer assembly

Refinements in the measurement of the heat capacity-temperature relation for aqueous dispersions of DMPC (dimyristoylphosphatidylcholine) yielded a critical temperature T* of 29 degrees, in agreement with previous film balance studies of this material. The transformation from multilamellar (multibilayer) to the critical unilamellar state commences at 28.8 degrees. Thus, the transformation occurs over a range of 0.2 degrees, a response that is typical of a second-order phase transition. The integrated heat over the temperature range of the transition is 2 mcal/gram of lipid. This corresponds to the work of separating bilayers in the multilamellar state to the critical, unilamellar state. This value is in reasonable agreement with estimates from osmotic stress studies of DMPC dispersions. (Gershfeld, Koshinuma)

Evidence for a lipid defect in Alzheimer's disease

As part of a continuing effort to test the validity of the critical bilayer theory as it pertains to membrane instability, critical temperatures T* for membrane lipids extracted from normal and diseased neurological tissues were examined. According to the theory if T* is below the physiological temperature, the membranes are unstable and the cells will degenerate. For cerebral cortex lipids from three Alzheimer's disease (AD) patients T* ranged from 19 to 28 degrees, independent of membrane protein composition. In contrast, control cortex lipids and cerebellar lipids from the AD patients yielded a normal value of 37 degrees. Thus, neuro-degeneration in AD may be explicable by membrane destabilization due to a lipid defect. Preliminary lipid analysis indicates a significant deficit of plasmalogen in AD membrane lipids compared to control membranes. (Gershfeld, Rapoport)

Platelet Aggregation

The volume increase of human platelet rich plasma due to DIPA (decompression-inducible platelet aggregation) is approximately 0.3% (v/v) or 3 ml per liter of packed human platelets. A similar volume increase during platelet aggregation was found when agonists such as epinephrine (adrenalin) and ADP were used; the volume increase was accompanied by a temperature drop. (Murayama)

Radiation Inactivation Analysis

This technique continued to generate interesting data in a wide range of biological systems.

Energy transfer between non-covalently associated polypeptides

The nature of energy transfer along and between polypeptides is of fundamental interest in the understanding of radiation damage to macromolecules. In general, radiation energy deposited in a single polypeptide is confined to that peptide, resulting in severe structural damage. If that peptide is joined by a disulfide bridge to another polymer, the radiation damage appears in both polypeptides. There are, however, seven reports of energy transfer between non-covalently associated peptides; the mechanism of such radiation energy transfer is unknown. If the transfer route is via the non-covalent bonds, it might be expected that the stronger the bonds the more efficient the energy transfer would be. The strongest non-covalent association known occur in the avidin-biotin interaction. Accordingly, a radiation inactivation study was undertaken of this system. In order to easily detect radiation damage on both sites of the interaction units, biotin was coupled to either glucose-6-phosphate dehydrogenase or to phycoerythrin and avidin was covalently tagged with fluorescent ligands or streptavidin was coupled to peroxidase. No difference in target size was seen when the derivatized biotins and avidins were irradiated independently or when coupled to each other. In this test system, no evidence could be found for radiation energy transfer across very strong non-covalent interactions. Thus the reports of inter-polypeptide transfer must be due to other unknown mechanisms, perhaps involving unique conformational associations. (Kempner)

Glutathione S-transferase

Glutathione S-transferase occurs both as a microsomal and a soluble enzyme, but they are both structurally and genetically different proteins. The soluble activity is due to a spectrum of related enzyme molecules which have been grouped into families distinguished by range of substrates and structure. Representative members of the alpha and mu families were irradiated and target size analysis revealed a important distinction between the two groups. Although the individual monomers of both families were destroyed one at a time (i.e., radiation energy deposited in one polypeptide destroyed only that polypeptide), the target size based on activity differed. The alpha family members require two subunits for activity while the mu members require only one. (Kempner)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27000-29 LPB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
The Mechanism of Muscular Contraction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard J. Podolsky	Chief	LPB/NIAMS
	Robert Horowits	Senior Staff Fellow	LPB/NIAMS
	Hiroyuki Iwamoto	Visiting Associate	LPB/NIAMS
	Sergey Malinchik	Visiting Associate	LPB/NIAMS

COOPERATING UNITS *(if any)*
Dr. Alasdair Steven, LSBR,NIAMS; Dr. Ellis Kempner, Section on Macromolecular Biophysics, LPB; Dr. Neal Epstein, NHLBI; Dr. Brian Collett, Hamilton College, Clinton, NY.

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Muscle Biophysics

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.5	3.5	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Striated muscle cells consist of three sets of filaments: (1) thick, myosin containing filaments, which interdigitate with (2) thin, actin-containing filaments and (3) titin filaments, which center the thick filaments axially in the sarcomere and produce the resting tension. The resting tension is different in fibers taken from different muscles: the tension produced by individual rabbit soleus muscle fibers is about five times smaller than that produced by rabbit psoas muscle fibers. The influence of sarcomere length on the movement of thick filaments away from the center of the sarcomere, which depends on the elastic properties of the titin filaments, shows that the titin filaments are stiffer in psoas fibers than in soleus fibers. Therefore the different tension levels in soleus and psoas fibers are due to a quantitative difference in the elastic properties of the titin molecules in the different muscle fibers.

The discovery that the weakly-binding actomyosin crossbridge has a different structure than the strongly binding crossbridge raised the possibility that physiological contraction is driven by a transition between these two states. This does not appear to be the case, however, because the force developed when nonhydrolyzable ATP analogues are first added to and then removed from covalently crosslinked muscle fibers depends on the level of preapplied stretch: no force is produced if the fiber is not prestretched. When the same muscle fiber is activated by calcium in an ATP-containing solution, larger forces are produced which are independent of the prestretch level (up to 1.5% muscle length). Thus the weak-strong crossbridge transition is not likely to be the physiological force-generating mechanism.

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MAJOR FINDINGS:

1. In the course of previous work, single fibers taken from human biceps or quadriceps muscles were found to have up to five times lower resting tension upon passive stretch than that produced by stretching rabbit psoas muscle fibers. Since proximal limb muscles were used in the human muscle study, limb muscles of the rabbit were used to see if they showed low levels of resting tension similar to those found in the human muscle fibers. Rabbit soleus muscle fibers turned out to exhibit very low levels of resting tension, similar to that previously found in the human fibers.

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2. Previous equatorial X-ray diffraction studies have shown that a myosin head covalently crosslinked to actin in a rigor muscle becomes rotatable upon addition of ligands like ATP, ATPγS (a nonhydrolyzable analog of ATP) or pyrophosphate. To know how this rotation of myosin head is related to the mechanism of muscle contraction, a reverse-type experiment was carried out: a crosslinked muscle was brought into rigor by the removal of ATPγS from the bathing solution and the changes in force, stiffness and equatorial diffraction pattern were observed. This procedure follows the sequence of an actomyosin ATPase cycle, in which ATP dissociates from myosin after hydrolysis. Upon removal of ATPγS the crosslinked muscle produced force in proportion to the level of preapplied stretch (up to 1.5% muscle length), but no force was produced if the muscle had not been prestretched. At any prestretch level, the muscle stiffness nearly doubled and the equatorial pattern was fully converted from relaxed- to rigor-type patterns. When the same muscle was activated by calcium in an ATP containing solution, it produced much larger force, independent of the prestretch level, while its stiffness and equatorial pattern were intermediate between those under relaxed and rigor conditions. Proposed mechanisms to explain

the results are as follows: (1) Myosin ligands confer rotatability to a myosin head by increasing its elasticity. (2) A rigor head and a head with a ligand have the same equilibrium orientation. Only when it has been prestrained does a myosin head exert force upon removal of ligand, by rotating back to its equilibrium orientation. (3) Distinct features of active contraction suggest the presence of another, as yet uncharacterized, force-producing mechanism. The role of observed head rotation may be to support or augment the force already present.

3. In previous studies the structural properties of rigor muscle fibers were studied at low ionic strength by X-ray diffraction and light microscopy. A puzzling observation was that at low Mg^{++} the fiber diameter expanded more than would be expected from the increase in filament lattice spacing measured by d_{11}. The explanation of this effect came from electron micrographs of fiber cross-sections fixed at low ionic strength. It was noted that while the thick filaments dissociate into many subfilaments, the thin filaments remain intact. However the arrangement of thin filaments is no longer strictly hexagonal; at low ionic strength the hexagons formed by the thin filaments are stretched so that some of the lattice planes are closer together than others, which tends to reduce the lattice spacing relative to the fiber volume. The cause of this unusual non-isotropic pattern of thin filament lattice expansion will be the subject of further study.

Project Description:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.
3. To characterize the structures and processes responsible for the high degree of order seen in striated muscle fibers.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.

2. Conventional and cryoelectronmicroscopy of muscle fibers fixed under various conditions.

3. Analysis of mechanical and structural properties of muscle fibers following selective degradation of the megadalton muscle proteins (titin and nebulin) by high energy radiation and/or enzymatic digestion.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of molecular mechanism of muscular contraction, together with the chemistry of the activation process, is useful in the rational handling of musculoskeletal, cardiovascular and arthritic diseases.

Proposed Course of Project:

1. The study by X-ray diffraction of the influence of stress on crossbridge configuration in the rigor state will be continued with rabbit psoas muscle. At present, these experiments are being done using a laboratory X-ray source. In the future, it is planned to make use of a brighter source (e.g., the National Synchrotron Light Source at Brookhaven, NY) which would significantly shorten the amount of time needed for data collection.

2. The structural changes associated with muscle contraction will be studied by cryoelectronmicroscopy and image processing, using techniques worked out by Trus et al. (1989).

3. The role of titin filaments in the binding of C-protein to thick filaments will be studied in rabbit muscle fibers by radiation inactivation analysis.

4. The influence of genetic point mutations in myosin heavy chain on the physiological properties of muscle fibers (force, velocity, etc.) will be examined in slow skeletal muscle obtained by biopsy from patients with hypertrophic cardiomyopathy. The experimental plan is based on evidence that the same myosin heavy chain appears in both cardiac myosin and slow muscle myosin.

Publication:

Podolsky RJ, Horowits R, Tanaka H. Ordering mechanisms in striated muscle fibers. In Frontiers of Muscle Research, Ozawa, E ed, Amersterdam Elsevier 1991;275-288.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27001-17 LPB

PERIOD COVERED: October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Regulation and Contractility of Skeletal and Smooth Muscle

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Schoenberg,	Medical Officer,	LPB, NIAMS
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	S. Qian,	IRTA Fellow,	LPB, NIAMS
	A. Ehrlich,	Biologist,	LPB, NIAMS

COOPERATING UNITS (if any)

LAB/BRANCH: Laboratory of Physical Biology

SECTION

INSTITUTE AND LOCATION: NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In FY91 our study of the myosin crossbridge interaction with actin in muscle fibers focused mainly on the strongly-binding crossbridge interaction. I derived a quantitative model of that interaction and tested the predictions of the model by studying the effect of ionic strength on the magnesium pyrophosphate crossbridge. It was found that increasing ionic strength from moderate values (~ 100 mM) to high values (~ 200 mM) has three significant effects. It causes a big decrease in the half time for the force decay after a small stretch, it causes a significant decrease in the sigmoidicity of the magnesium pyrophosphate concentration dependence of the apparent rate constant of force decay after a small stretch, and it causes a big decrease in the range of rate constants necessary to describe the multiexponential force decay. It causes the last of these by causing a much larger increase in the slowest rate constants of the decay than in the fastest rate constants. The results offer strong support for our proposed model of the actin - strongly-binding crossbridge interaction.

In smooth muscle we examined the relationship between myosin light chain phosphorylation and force during atrial natriuretic factor (ANF) induced relaxation. Unlike the situation for the onset of contraction, the two do not correlate closely. When ANF relaxes a KCl-induced contraction, a 50% reduction in myosin light chain phosphorylation is accompanied by only a 30% reduction in force. When ANF relaxes a 10^{-7} M acetylcholine contraction, there is no detectable change in myosin light chain phosphorylation but a 50% decrease in force. There is no explanation for these findings at present, especially since we found that ANF applied to a relaxed fiber produces no change in either force or myosin light chain phosphorylation.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Z01 AR 27001-17 LPB

OBJECTIVES: To understand the molecular basis of muscle contraction.

METHODS AND MAJOR FINDINGS:

I) Contractility of skeletal muscle: Interaction of strongly-binding crossbridges with actin

In recent years, our main contribution has been elucidation of the behavior of weakly-binding crossbridges. The interaction of weakly-binding myosin crossbridges with actin was previously unrecognized and elucidation of that behavior revealed many new aspects of the actin - myosin interaction. Most of the insights gained were applicable to strongly-binding crossbridges myosin crossbridges as well. However, while these insights provided valuable qualitative information about the behavior of strongly-binding bridges, quantitatively our understanding of the interaction of strongly-binding crossbridges with actin remained incomplete. In FY91, this difficulty was resolved using a two-fold approach which included development of a quantitative model for the strongly-binding crossbridge - actin interaction and completion of a number of experiments testing that model.

The myosin crossbridge has two subfragment-1 heads that can bind to actin. With the weakly-binding crossbridge interaction these two heads appear to function independently. For the strongly-binding intereaction, this is no longer the case. In our model of the strongly-binding interaction, the binding of one crossbridge head severely restricts the allowable sites for the binding of the second head. This has a very big influence on the behavior of the crossbridge, particularly its ability to sustain force when strained. This predicted influence can be examined by using ionic strength to change the strength of the binding of the crossbridge heads. In FY91 it was found that increasing ionic strength from moderate values (μ ~ 100 mM) to high values (μ ~ 200 mM) has three effects. It causes a big decrease in the half time for the force decay after a small stretch, it causes a significant decrease in the sigmoidicity of the nucleotide analogue concentration dependence of the "apparent rate constant" of force decay after a small stretch, and it causes a big decrease in the range of rate constants necessary to describe the multiexponential force decay. It causes the last of these by causing a much larger increase in the slowest rate constants of the decay than in the fastest rate constants. The results offer strong support for our proposed model of the actin - strongly-binding crossbridge interaction.

II) Contractility of skeletal muscle: Binding of myosin·ATP crossbridges and temperature

In order to reduce the likelihood of a relaxed fiber becoming active, the initial experiments on weakly-binding myosin·ATP crossbridges were done at 5°C. In FY91 we showed that myosin·ATP crossbridges were observable at 20°C. Significantly, the Q_{10} temperature coefficient for crossbridge binding suggests that the weakly-binding myosin·ATP crossbridges are likely also to be found at 38°C, the body temperature of the living rabbit.

II) Regulation of smooth muscle

uring the onset of contraction in smooth muscle, there is a reasonable correlation etween myosin light chain phosphorylation and force development. Ca^{2+}-calmodulin ependent activation of myosin light chain kinase appears to lead to phosphorylation f the 20 kDalton myosin light chain which stimulates the myosin crossbridges to roduce force. In FY90 we developed a sensitive radio-assay for detecting the ercentage of 20-kDalton myosin light chain phosphorylation in small preparations of racheal smooth muscle. In FY91 we used this assay to correlate myosin light chain hosphorylation and force production during atrial natriuretic factor (ANF) induced elaxation. Surprisingly, a large discrepancy between force and myosin light chain eduction in myosin light chain phosphorylation was found. When ANF relaxes a KCl-nduced contraction, a 50% reduction in myosin light chain phosphorylation is ccompanied by only a 30% reduction in force. When ANF relaxes a 10^{-7} M acetylcholine ontraction, there is no detectable change in myosin light chain phosphorylation but 50% decrease in force. There is no explanation for these findings at present, specially since we found that ANF applied to a relaxed fiber produces no change in ither force or myosin light chain phosphorylation.

IGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

xperiments on the mechanism of muscular contraction are important in that they ontribute to a sound understanding of how normal muscle works. This, in turn, is seful in understanding diseases muscle. Furthermore, the high degree of organization f the contractile proteins in skeletal muscle makes this a particularly useful system or elucidating contractile mechanisms, some of which may be common to the cellular and ubcellular motions of other, less easily studied, cells. Understanding the regulation f smooth muscle of course, likely holds the answer to any number of important uestions, one of them being the etiology of essential hypertension.

UTURE COURSE:

e hope that our enhanced understanding of both the beginning and end of the rossbridge power stroke will enable use to better understand the force-producing power troke itself. We also hope to examine in more detail what happens when weakly-binding rossbridges are produced using pPDM or NPM treatment. Of particular interest is ocating the site (s) and stoichiometry of reactivity.

UBLICATIONS:

orson MA, Sellers JR, Adelstein RA, Schoenberg M. Substance P contracts bovine racheal smooth muscle via activation of myosin light chain kinase, AM J Physiol 990;259:C258-C265.

ajer PG, Fajer ER, Schoenberg M, Thomas DD. Orientational disorder and motion of eakly attached crossbridges, Biophys J 1991; in press.

choenberg M. Equilibrium muscle crossbridge behavior: theoretical considerations II. model describing the behavior of strongly-binding crossbridges when both heads of yosin bind to the actin filament, Biophys J 1991; in press.

choenberg M. Effect of ionic strength on skinned rabbit psoas fibers in the presence f magnesium pyrophosphate, Biophys J 1991; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 27003-32 LPB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Biophysical Studies of Metabolic Activity and Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Ellis S. Kempner, Ph.D., Physicist and Chief,
Section on Macromolecular Biophysics LPB NIAMS

COOPERATING UNITS *(if any)*

Drs. T. Boyer (Emory Univ.), J. Straka and J. Bloomer (Univ. Minnesota), Dr. David Via (Baylor Univ.).

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0.	1.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Target analysis of the inactivation of biological activity by ionizing radiation was applied to several major problems including the microsomal glutathione S-transferase, mitochondrial ferrochelatase, and the acetyl-LDL receptor. Fundamental studies of radiation energy transfer was examined in the avidin-biotin system.

Objectives: 1) An understanding of the nature of active structures in vivo which are involved in biochemical processes, principally by means of the technique of inactivation by ionizing radiation.
2) Detailed knowledge of the molecular damage caused by ionizing radiation and of the mechanisms of the transfer of radiation energy throughout these structures.

Methods: 1) General biochemical techniques including enzyme reactions, fluorescence, and gel electrophoresis.
2) Ionizing radiation, usually high energy electrons from a linear accelerator, to expose samples under carefully controlled conditions.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

Major findings:

Glutathione S-transferase occurs both as a microsomal and a soluble enzyme, but they are both structurally and genetically different proteins. The soluble activity is due to a spectrum of related enzyme molecules which have been grouped into families distinguished by range of substrates and structure. Representative members of the alpha and mu families were irradiated and target size analysis revealed an important distinction between the two groups. Although the individual monomers of both families were destroyed one at a time (i.e., radiation energy deposited in one polypeptide destroyed only that polypeptide), the target size based on activity differed. The alpha family members require two subunits for activity while the mu members require only one.

Ferrocheletase is the terminal enzyme in heme synthesis, catalyzing the insertion of iron (II) into protoporphyrin. It is an integral protein of the inner mitochondrial membrane. The functional size of bovine hepatic ferrochelatase was studied in situ using radiation inactivation analysis. The functional unit required for enzymatic activity in intact mitochondria was found to have a mass of 82 kDa. In contrast the structural unit (evaluated in immunoblots following SDS-polyacrylamide gel electrophoresis) has a mass of 40 kDa. Similar results were obtained when irradiation was performed on sodium cholate-solubilized mitochondria. The presence or absence of DTT during irradiation had no effect on target sizes obtained from either intact or solubilized mitochondria. Previous studies using SDS-PAGE and gel filtration chromatography was shown that an Mr 40,000 peptide is associated with ferrochelatase activity. The results of the radiation study show that bovine ferrochelatase activity requires two interacting subunits of 40 kDa each, and explains the ferrochelatase activity level observed in hepatic tissue of hetero- and homozygous protoporphyric animals.

The functional molecular size of the macrophage receptor for acetyl low density lipoprotein (Ac-LDL) was determined directly in membranes by radiation inactivation analysis. Membranes from tumors induced by macrophage cell line P388D1 were frozen and irradiated with high energy electrons. Analysis of residual binding activity indicated a functional molecular mass of 35 kDa, considerably smaller than the active 260,000 Mr protein seen on ligand blots under non-reducing conditions. Scatchard analysis of receptor binding gave no evidence of partially inactivated molecules. The receptor protein, further purified by affinity chromatography and preparative gel electrophoresis, was incubated with DTT (0.1 - 100 mM) and retested for binding activity. Active subunits of 158,000 Mr and 80,000 Mr could be demonstrated by ligand blotting, with quantitative conversion to the 80,000 Mr species at 10 mM DTT. At 100 mM DTT and greater all binding activity was lost. Further size reduction was not detected by silver staining. These data suggest that the mouse macrophage Ac-LDL receptor as isolated is trimeric in nature with one class of SH groups involved in trimerization and another in the actual binding site, and that the monomeric species is fully active in vitro under mild reducing conditions. In addition, the radiation inactivation data suggest that each monomeric unit is fully active and capable of functioning independently in the binding of ligands in the membrane.

The nature of energy transfer along and between polypeptides is of fundamental interest in the understanding of radiation damage to macromolecules. In general, radiation energy deposited in a single polypeptide is confined to that peptide, resulting in severe structural damage. If that peptide is joined by a disulfide bridge to another polymer, the radiation damage appears in both polypeptides. There are, however, seven reports of energy transfer between non-covalently associated peptides; the mechanism of such radiation energy transfer is unknown. If the transfer route is via the non-covalent bonds, it might be expected that the stronger the bonds the more efficient the energy transfer would be. The strongest non-covalent association known occur in the avidin-biotin interaction. Accordingly, a radiation inactivation study was undertaken of this system. In order to easily detect radiation damage on both sites of the interacting units, biotin was coupled to either glucose-6-phosphate dehydrogenase or to phycoerythrin and avidin was covalently tagged with fluorescent ligands or streptavidin was coupled to peroxidase. No difference in target size was seen when the derivatized biotins and avidins were irradiated independently or when coupled to each other. In this test system, no evidence could be found for radiation energy transfer across very strong non-covalent interactions. Thus the several reports of inter-polypeptide transfer must be due to other unknown mechanisms, perhaps involving unique conformational associations.

Future course:

Two lines of research will be continued. Fundamental studies of radiation include a) the nature of energy transfer between and along polymers; b) examination of radiolytic fragments from proteins of known structure; and c) examination of conformational changes in irradiated macromolecules. Applications of target analysis to important biochemical problems includes phospholipases, phenylalanine hydroxylase, and cocaine receptors.

Publications:

Kempner ES, Miller JH. Direct effects of radiation on the avidin-biotin system: absence of energy transfer, J Biol Chem 1990;265:15776-15781.

Kempner ES, Osborne Jr JC, Reynolds LJ, Deems RA, Dennis EA. Analysis of lipases by radiation inactivation, Methods in Enzymol 1991;197:280-288.

In press:

Boyer TD, Kempner ES. Effect of subunit interactions on enzymatic activity of glutathione s-transferases: a radiation inactivation study.

Straka JG, Bloomer JR, Kempner ES. The functional size of ferrochelatase determined in situ by radiation inactivation.

Via DP, Kempner ES, Pons L, Fanslow AE, Vignale S, Smith LC, Gotto Jr AM, Dresel HA. Mouse macrophage ac-ldl receptor: demonstration of a fully functional subunit in the membrane and with purified receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27004-22 LPB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Dynamic Properties of Cell Membranes and Related Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Norman L. Gershfeld, Ph.D.	Research Chemist LPB,NIAMS
	Masakatsu Koshinuma, Ph.D.	Visiting Scientist
	William F. Stevens, Jr.	Biological Lab Technician

COOPERATING UNITS *(if any)*

Dr. Courtney P. Mudd, ACES, BEIP, NCRR
Dr. S. I. Rapoport, LN, NIA

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Laboratory of Physical Biology

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Aqueous suspensions of phospholipids, either synthetic or natural mixtures from cell membranes, have been used to study how membrane bilayers assemble. The studies are based on a theory which describes membrane bilayer assembly as a physicochemical process that occurs only at a critical point, the physiological temperature of the cell. The optimum conditions for membrane bilayer stability, a prerequisite for normal cellular function, occur at the critical point; membrane lipid composition and the critical temperature are interdependent. When either lipid composition or temperature deviate from the required critical conditions, the membrane bilayer becomes unstable and its structure degenerates with catastrophic consequences for the cell. This mechanism of pathogenesis has been successfully tested with neural tissue lipids from a patient with metachromatic leukodystrophy, a disease with a well-defined lipid defect. Thus, measurements of the critical conditions for membrane bilayer assembly appear to be relevant for identifying membrane lipid defects in cellular dysfunction. The relevance of this membrane destabilization mechanism in other diseases with obscure etiologies is presently being tested.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

Objectives:
The present goals of this project are to describe how membrane bilayers assemble and to identify conditions which cause bilayer instability and membrane breakdown. The studies are based on a theory which describes bilayer assembly as a physicochemical process that occurs at a critical point, the physiological temperature of the cell; membrane lipid composition and the critical temperature are interdependent. According to the theory, the optimum conditions for membrane bilayer stability, a prerequisite for normal cellular function, exist at the critical point. When either the membrane lipid composition or ambient temperature is altered, the theory indicates that the bilayer will degenerate followed by cellular destruction. To test this mechanism of pathogenesis total membrane lipids from dysfunctional neurological tissues have been examined.

Methods:
A) Quasi-Adiabatic Scanning Microcalorimetry
This calorimeter has been designed to obtain critical bilayer assembly temperatures from measurements of the heat capacity of membrane lipid dispersed in water. First described in the 1990 Annual Report, this instrument has undergone a number of substantial changes in the design of the cells, in the computer software for controlling the temperature of the calorimeter and for data collection. These changes permit easier loading of the cells and provide greater sensitivity of the heat capacity measurement.

B) Quantitative Analysis of Membrane Lipids
An important aspect of lipid bilayer assembly is to identify lipid defects that may cause bilayer instability. Thus, we continue to develop methods for quantitatively analyzing all membrane phospholipids. One of the lipids that has frequently been implicated in a number of diseases is the plasmalogen of phosphatidylethanolamine (PPE). A new method for the quantitative analysis of these plasmalogens has been developed. It is based on separating the plasmalogen from its mixture with PE by reaction with HCl. The plasmalogen is selectively converted to the lyso compound which can then be separated from PE by thin layer chromatography.

Major findings:
A) Calorimetry of critical bilayer assembly
Refinements in the measurement of the heat capacity-temperature relation for aqueous dispersions of DMPC (dimyristoylphosphatidylcholine) yielded a critical temperature T* of 29 degrees, in agreement with previous film balance studies of this material. The transformation from multilamellar (multibilayer) to the critical unilamellar state commences at 28.8 degrees. Thus, the transformation occurs over a range of 0.2 degrees, a response that is typical of a second-order phase transition. The integrated heat over the temperature range of the transition is 2 mcal/gram of lipid. This corresponds to the work of separating bilayers in the multilamellar state to the critical, unilamellar state. This value is in reasonable agreement with estimates made from osmotic stress studies of DMPC dispersions.

B) Evidence for a lipid defect in Alzheimer's disease
As part of a continuing effort to test the validity of the critical bilayer theory as it pertains to membrane instability we have been examining critical temperatures T* for membrane lipids extracted from normal and diseased neurological tissues. According to the theory if T* is below the physiological

temperature, the membranes are unstable and the cells will degenerate. We have previously found this pathogenetic mechanism to be applicable in metachromatic leukodystrophy, a disease with a known lipid metabolic defect, and have therefore measured T* for brain tissue with Alzheimer's disease whose etiology is presently in dispute. For cerebral cortex lipids from three AD patients T* ranged from 19 to 28 degrees, independent of membrane protein composition. In contrast, control cortex lipids and cerebellar lipids from the AD patients yielded a normal value of 37 degrees. Thus, neuro-degeneration in AD may be explicable by membrane destabilization due to a lipid defect. Preliminary lipid analysis indicates a significant deficit of plasmalogen PE in AD membrane lipids compared to control membranes.

Publications:

Ginsberg L, Gilbert DL, Gershfeld NL. Membrane bilayer assembly in neural tissue of rat and squid as a critical phenomenon: influence of temperature and membrane proteins, J Membr Biol 1991;119:65-73.

In press:

Ginsberg L, Gershfeld NL. Membrane bilayer instability and the pathogenesis of disorders of myelin. Neuroscience Letters.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 27005-09 LPB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Aggregation of Human Platelets Induced by Decompression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Makio Murayama Research Chemist LPB, NIAMS

COOPERATING UNITS *(if any)*

Dr. K.K. Kumaroo, Biochemist, U.S. Naval Research Institute, Bethesda, MD

LAB/BRANCH

Laboratory of Physical Biology

SECTION

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INSTITUTE AND LOCATION

NIAMS, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The main thrust of the laboratory is focussed on the molecular mechanism of platelet aggregation, including both hemostasis and thrombosis, investigating the influence of pressure on the hydration layer surrounding the electrically charged amino acid residues involved in platelet aggregation. We have been investigating the molecular mechanism of platelet interactions in DIPA (decompression-inducible platelet aggregation) for the past several years. We have found that the oppositely charged amino acid residues, i.e., positively charged arginyl and negatively charged aspartyl residues interact with their respective receptor sites. Last year we observed vascular occlusion in the small blood vessels in the web of the frog's foot and in the ear of the mouse. During the current year we have found that the volume of human PRP is increased by 0.3% or 3 ml per liter of packed thrombocyte volume when platelet aggregation is induced by decompression. A similar increase in the volume of human PRP is now being observed when platelet aggregation is induced by the agonists, epinephrine (adrenalin) and ADP. We theorize that compactly organized water molecules, when randomized into the bulk phase, acquire thermal motion which causes a temperature drop; and that human platelet aggregation is an entropy driven process similar to human red cell sickling. To confirm our hypothesis, we are utilizing a newly designed dilatometer which has a "built-in" thermistor, to measure the volume increase and temperature drop when platelet aggregation is induced by epinephrine (adrenalin) and ADP.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Major Findings:

During the previous year we concluded that acute mountain sickness is a vascular occlusive disease. We found that vascular occlusion could be prevented in the mouse as well as in the frog by the oral administration of the drug piracetam. We also found that thymol, and other compounds can prevent DIPA in the frog and mouse. I had discovered almost a decade ago that decompression can be substituted for agonists to study platelet aggregation. During the past year we determined that the volume increase of human PRP due to DIPA (decompression-inducible platelet aggregation) is approximately 0.3% (v/v) or 3 ml per liter of packed human platelets. We found a similar volume increase during platelet aggregation when agonists such as epinephrine (adrenalin) and ADP were used; the volume increase was accompanied by a temperature drop.

Project Description:

Objectives: The main objectives are to investigate the molecular mechanism underlying platelet aggregation using decompression as an additional agonist and the mode of action of inhibitors of platelet aggregation. Platelet aggregation is fibrinogen-dependent; any mechanism which can block fibrin (ogen) aggregation could also block the initial stages of human platelet aggregation. This can be done by neutral salts and also (at a lower concentration) by a molecule having a large electric dipole moment such as the drug piracetam, which blocks fibrin polymerization and inhibits platelet aggregation. It is now generally accepted that the amino acid residues involved in the specific recognition site on the platelet membrane and in the binding site of fibrin (ogen) is the peptide sequence Arg.Gly.Asp (RGD). We have shown that RGD causes 50 per cent inhibition of platelet aggregation in DIPA (decompression-induced platelet aggregation). Therefore, it is hypothesized that electrical charges are involved in platelet aggregation. The positive charge resides in the Arginine residue and the negative charge in the carboxyl group of Aspartic acid, indicating that electrostatic interaction is the primary mechanism of platelet aggregation. Electrical charges are "insulated" by compactly organized water molecules radially surrounding the charges; the bound water must be released to permit interaction of positive and negative charges. Therefore it is assumed that the platelet aggregation is accompanied by a volume increase of the solution (plasma) and a concomitant temperature drop. To provide experimental evidence to investigate this aspect of our objective we are using a specially designed dilatometer with a built in thermistor.

Methods Employed:

Dilatometry is used to measure the volume change of human PRP due to DIPA and platelet aggregation due to agonists such as epinephrine (adrenalin), ADP, and collagen. The dilatometric method measures volume changes produced by a system as a consequence of physical and/or chemical change. These volume changes reflect changes in the partial molar volume of some or all of the components in the system. To measure the volume increase in human PRP due to DIPA, a simple dilatometer was fabricated in the NIH Glass Shop. The simple dilatometer consists of a round bottomed flask with a capacity of 97 ml. having a neck of 2 mm precision bore capillary tubing 14 cm long.

The dilatometer is filled with concentrated PRP, up to 10 mm of the capillary tubing above the flask. The packed cell volume (thrombocrit) is 15 to 25 percent (vol/vol). Sufficient mineral oil is added to fill the capillary completely. The volume increase that results from DIPA is determined by the change in the height of the plasma-oil meniscus before and after the aggregation of the human platelets. The height of the plasma-oil meniscus in the capillary tubing is determined with a cathetometer, after obtaining thermal equilibration with the initial height and final height, finally calculating the volume increase for packed human thrombocytes (platelets). The volume increase that results from agonists such as epinephrine, ADP and collagen are measured by using the classical "Y"-tube dilatometer having a "built-in" thermistor to measure the temperature change as well as the volume increase.

Significance to Biomedical Research and the Program of the Institute:

The platelet is an excellent model to understand how cells adhere to each other. The platelet-to-platelet adhesion mechanism is now believed to be similar to platelet-to-endothelial cell adhesion. The underlying molecular mechanism of this adhesion process is essential in understanding vascular occlusive diseases such as acute mountain sickness (AMS), diver's diseases (DCS), heart attack and stroke and their prevention/amelioration. It is important to confirm the basic assumption of DIPA: there is a volume increase of human PRP during DIPA. it was assumed that there is a volume increase when electrically charged groups interact, thus neutralizing the opposite charges. This produces concomitant relaxation of electrostriction, accompanied by randomization of the organized water molecules surrounding the charged residues.

Proposed Course of Project:

We will continue to investigate the molecular mechanism of platelet aggregation utilizing DIPA and agonists such as epinephrine (adrenalin), ADP and collagen. In this stage of our experiments, we will continue to measure the volume increase during DIPA and the volume increase during platelet aggregation induced by agonists such as epinephrine (adrenalin), ADP, and collagen as well as the accompanying temperature drop. Further investigation of the mechanism of cell adhesion will be undertaken using the platelet as a model. Peptides related to RGD such as KQAGD will be used as a probe to study the inhibition of platelet aggregation (cell adhesion).

Publication:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 27012-07 LPB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural and Mechanical Properties of Muscle Fibers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Leepo C. Yu,	Research Physicist,	LPB, NIAMS
	Sengen Xu	Visiting Associate,	LPB, NIAMS
	Daniel Gilroy	Mathematician	LPB, NIAMS

COOPERATING UNITS *(if any)*

University of Ulm, FRG (Drs. B. Brenner and T. Kraft);
East Carolina University Medical School (Dr. J. Chalovich).

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Sections on Muscle Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.60	2.0	0.6

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The continuing focus of this project is to determine structural changes involved in contraction mechanism and to identify essential steps in the pathway of force generation.

It was shown earlier that at low ionic strength and low temperature an essential intermediate crossbridge state immediately preceding force generation is an attached state with low affinity between myosin and actin. New evidence shows that at near physiological conditions active force level is similarly inhibited if the binding of the myosin to actin is inhibited in the low affinity states. The results further confirmed the importance of the low affinity but attached crossbridge states in force production and the existence to a significant extent of such states in the relaxed muscle.

High resolution equatorial X-ray studies were carried out to investigate whether there are phase changes in transforming the conformation of the crossbridges from relaxed state to rigor state. Preliminary results suggest that one reflection, [3,0], may undergo a phase change. The differences in the conformation of the relaxed and the rigor crossbridges are probably more pronounced than previously proposed.

A new project has been initiated to investigate the role of the strongly bound crossbridge states in regulation and contraction of muscle by introducing exogenous myosin fragments modified by N-ethylmaleimide into the muscle fiber. The first phase of the project has determined the time course of binding of the myosin fragment to actin. Preliminary mechanical data suggest that the binding of such modified myosin fragment increases the sensitivity of Ca^{++} regulation and inhibits active force level.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description:
To understand muscle contraction mechanism by identifying and characterizing the essential steps in the pathway of force generation. The main focus of this project is to determine structural changes of the contractile proteins involved in force production.

Methods Employed:
Vertebrate skeletal muscle fibers are demembranated such that the medium bathing the contractile proteins is directly intervened, such that various contraction states can be achieved or simulated. X-ray diffraction from the muscle fibers under various intervention are used to study the structure of the contractile proteins. The structural results are correlated with mechanical and biochemical properties of the muscle fibers and muscle proteins, such that various structural states are correlated with functions of muscle.

Major Findings:
(1) It has been shown that the actomyosin (crossbridges) ATP hydrolysis cycle can be divided into two groups of states: the weak binding states characterized by low affinity between actin and myosin and the strong binding states characterized by strong affinity. Since the discovery of its existence in muscle fibers, the physiological role of the weak binding crossbridge states has been a subject of debate. Last year we used caldesmon, a regulatory protein found in smooth muscle, to specifically inhibit crossbridge formation in the weak binding states. Inhibition of crossbridge formation in the weak binding states caused inhibition of active force. Therefore, the weak binding states are essential precursors to force generation. However, the conditions of the experiments were at low temperature and low ionic strength to facilitate the detection of the weak binding crossbridges.
Following last year's results, we now have obtained preliminary data at near physiological conditions: at temperature as high as 20° C and ionic strength as high as 170 mM. X-ray diffraction data shows that the actomyosin binding is decreased as concentration of caldesmon is increased; mechanically the fiber resting stiffness is reduced by caldesmon and more importantly, active force is inhibited. The extent of inhibition in active force level is very similar to that found at lower temperature and lower ionic strength. The results indicate that first, crossbridges do form to a significant extent in the weak binding states at near physiological conditions; secondly, inhibition of crossbridge attachment in the weak binding states is sufficient to inhibit active force. Therefore, there is now strong evidence that attached weak binding crossbridges are essential for force generation in skeletal muscle.

(2) Two dimensional electron density maps of muscle cells reconstructed from the first five equatorial X-ray diffraction intensities indicated that the conformations of the attached crossbridges in the relaxed (weak binding) and rigor (strong binding) states differ from each other. However, in reconstructing the density maps it was assumed that the phases of the X-ray reflections were the same in the two states. If there are changes in the phases, the difference in the density maps would be even more prominent. One way to test the assumption of phases being unchanged is to modulate the fraction of attached crossbridges in the relaxed state vs. in the rigor state. This was achieved by titrating the ATP analogue ATPγS in the bathing solution. Among the first five reflections, there is one reflection, [3,0], appears to be biphasic with increasing concentration of ATPγS. This raises the possibility of a phase change in [3,0]

as the population of the attached crossbridge shifts from the relaxed to rigor conformation.

(3) We have initiated a new study investigating the role of the strong binding crossbridges in regulation and force generation in muscle. In solution, the ATPase activity of myosin, in the presence of actin-tropomyosin-troponin, can be activated by Ca^{2+} or by the binding of rigor crossbridges. N-ethylmaleimide (NEM) modified S1 (myosin fragments) is used to investigate the role of strong binding crossbridges in muscle contraction. Since S1 is a large molecule (M.W. $\approx$ 120,000), as a first step, we have determined the time course of binding of the modified S1 in muscle fibers by monitoring the changes in the intensities of the X-ray reflections. Mechanical measurements reveal that the binding of the modified S1 to actin increases the sensitivity of Ca^{2+} regulation. At the same time, the modified S1 decreases the maximum level of active tension.

(4) We have further analyzed our data concerning the elastic behavior of the attached crossbridges in radial direction perpendicular to the fiber axis. Two parameters of the elasticity were examined. The equilibrium point where the radial force is zero is shown to be clearly a function of the state of the crossbridge. The other parameter, i.e. stiffness per crossbridge, is very likely also a function of the state of the crossbridge, although the evidence is not as direct. The difference in radial elasticity most likely reflects structural differences of the attached crossbridges. This approach of determining radial elasticity should prove to be an effective technique as a first screening for structural differences in attached crossbridges, since this approach does not depend on the number of crossbridges attached to actin, a factor frequently encumbers interpretation of other structural studies.

Future Plans:

(1) The role of the weak binding crossbridges and the strong binding crossbridges in regulation and force generation will be further investigated, particularly by introducing various agents, such as the NEM modified S1, into the muscle fibers.

(2) The two dimensional X-ray diffraction camera, with the expected arrival of an imaging plate detector system, will be used to study muscle structure.

Publications:

Brenner B, Yu LC, Chalovich, JM. Parallel inhibition of active force and relaxed fiber stiffness in skeletal muscle by caldesmon: Implications for the pathway to force generation, Proc Nat Acad Sci (USA) 1991;88:5739-5743.

Brenner B, Yu LC. Characterization of radial force and radial stiffness in Ca^{++} activated skinned fibres of the rabbit psoas muscle, J Physiol. (London) 1991; 441:703-718.

In Press:

Chalovich J, Yu LC, Velaz L, Kraft T, Brenner, B. Caldesmon derived polypeptides as probes of force production in skeletal muscle. In Peptide Competition Approaches in Muscle Research, ed. J. C. Rüegg. Springer-Verlag.

Chalovich J, Yu LC, Brenner, B. Involvement of weak binding cross-bridges in force production in muscle. J. Muscle Res. & Cell Motil.

ANNUAL REPORT

LABORATORY OF STRUCTURAL BIOLOGY RESEARCH
NATIONAL INSTITUTE OF ARTHRITIS, MUSCULOSKELETAL AND SKIN DISEASES

OCTOBER 1, 1990 THRU SEPTEMBER 30, 1991

Structure, Assembly, and Function of Biological Macromolecules

In the past year, the Laboratory of Structural Biology Research has entered on a transition phase involving a substantial turnover of key personnel, and a major commitment of time and other resources to upgrading our experimental facilities, and organizing an expansion of our program as a whole. Nevertheless, it has been possible to make substantial headway in several areas of investigation, and to initiate several other projects. The molecular architecture of the capsid of herpes simplex virus, type 1, has been studied by cryo-electron microscopy and three-dimensional image reconstruction of biochemically depleted and antibody - labelled capsids. Taken together, our data have led to a tentative localization of the four minor capsid proteins (VP19, VP22, VP23 and VP26). They suggest that VP22 is a transient form-determining core protein, and that VP19 and VP23 serve to stabilize the capsid by binding to its outer surface. Two new lines of evidence reinforce the view that the cooperative conformational change that accompanies maturation of the bacteriophage T4 capsid is a much more radical event than the conformational transitions previously characterized in such contexts as ligand-binding by allosteric enzymes. We have found that the T4 expansion transformation involves translocation of three distinct epitopes from the inner surface to the outer capsid surface, or vice versa. Moreover, the proteolytic cleavage event that normally precedes expansion has been found to result in a much stabler conformation than if expansion is induced without prior cleavage.

Herpesvirus Capsid Assembly.

Our long term objective is to work out the assembly pathway of this archetypal member of an extensive family of animal viruses, several of which have important associations with human diseases. We have found that treatment of purified capsids with 2.0M guanidine hydrochloride quantitatively removes VP26, the smallest capsid protein, and causes the penton capsomers to disappear. Upon removal of the denaturant by dialysis, VP26 reassociates with the capsid. and material reappears at the vertex sites, albeit with an altered morphology compared with the original pentons. Exposure of capsids to a monoclonal antibody against VP5 results in strong labelling of the hexons but no labelling of pentons. The epitope has been localized with high precision to the extreme distal tip of the hexons' external protrusions. These data imply that pentons are composed of some protein other than VP5, and are thus consistent with the tentative identification of VP26 as the penton protein on the basis of the Guan.HCl extraction experiments. Guanidine also releases a fraction of both VP19 and VP23, and causes the disappearance of most of the 120 peripentonal triplexes (there are 300 triplexes in all). Taking into account stoichiometry data, these observations tentatively suggest that the triplexes are heterotrimers consisting of one copy of VP19 and two copies of VP23. Our continuing experiments are aimed at testing and further refining this scenario by conducting similar experiments with other antibodies. (Booy, Trus, Newcomb, Brown, Conway, Steven).

Conformational Basis of Phage T4 Capsid Maturation.

The T4 capsid is first assembled as a relatively fragile, DNA-free, particle, into which DNA is subsequently packaged. After packaging, the mature capsid is a considerably larger (18%) and much more resistant structure. The basis of this transition is a concerted conformational change undergone by the ~1000 molecules of gp23 that are arrayed in its icosahedral surface lattice. We have continued our multi-disciplinary approach to characterize this exceptionally large-scale, irreversible, regulatory conformational change, and have completed two phases of this work over the past year. In the first, monospecific antibodies were raised against four synthetic peptides that correspond to defined segments of gp23, the major capsid protein. These antibodies were used to label the precursor (uncleaved, unexpanded) and mature (cleaved, expanded) states of the capsid surface lattice and examined by immuno-electron microscopy. The results demonstrate that at least three distinct peptides are translocated between the inner and the outer surfaces when expansion occurs. In the second study, differential scanning calorimetry was used to characterize the thermodynamic properties of two states that are intermediate between the precursor and the mature forms, viz. the cleaved unexpanded state (which occurs on the in vivo pathway). and the uncleaved, expanded state (which can be induced in vitro). The results provide a rationale for the cleavage reaction in that they demonstrate that a substantially greater stabilization accompanies expansion ($\Delta(\Delta T_m) \sim 10°C$) if it is preceded by full cleavage of gp23 to gp23*. (Steven, Greenstone, Booy, Bauer, Bisher. Black, Ross).

Molecular Structure of Clathrin, the Coating Protein of Endocytotic Membranes.

There is an extensive class of oligomeric fibrous proteins which typically contain several copies of a very large molecular weight subunit in association with several smaller subunits. Such molecules are particularly prevalent in the cytoskeleton and the extracellular matrix. Moreover, they often flexible and exhibit curvature that renders them inaccessible to conventional methods of structural analysis. We have developed image averaging techniques specifically designed for analysis of electron micrographs of such molecules. These techniques, which involve straightening by cubic-spline interpolation, image classification, and statistical analysis of the molecules' curvature properties, have been applied to purified brain clathrin. This trimeric filamentous protein polymerizes, both in vivo and in vitro, into a wide range of polyhedral structures. Contrasted by low-angle rotary shadowing, dissociated clathrin molecules appear as distinctive three legged structures, "triskelions". We find triskelion legs to vary from 35 nm to 62 nm in total length. Peaks in averaged curvature profiles mark hinges or sites of enhanced flexibility. Such profiles, calculated for each length-class, show that three curvature peaks are observed in every case: their locations define a proximal segment of systematically increasing length (14.0 - 19.0 nm); a mid segment of fixed length (~12 nm); and a rather variable end segment (11.6 - 19.5 nm), terminating in a hinge just before the globular terminal domain (- 7.3 nm diameter). Thus, two major factors contribute to the overall variability in leg-length: (1) stretching of the proximal segment and (2) stretching of the end segment and/or scrolling of the terminal domain. The observed elasticity of the proximal segment may reflect phosphorylation of the clathrin light chains. (Kocsis, Trus, Steer, Bisher, Steven).

Polymerization of the REV Protein of Human Immunodeficiency virus.

Rev is a regulatory protein which plays an important role in the phase of infection in which massive production of virus takes place. It appears that rev acts by preventing splicing of the mRNA for the env glycoprotein, leading to the successful export of this mRNA from the nucleus and ensuing synthesis of the glycoprotein. We have studied the assembly properties of rev obtained from an E. coli expression system. Purified rev has the remarkable property of self-assembling in vitro into long, hollow, uniformly wide (20nm) filaments which bear a striking resemblance to helical polymers of tobacco mosaic virus coat protein. We are seeking to determine the structure of these filaments by means of electron microscopy and image processing. We have also discovered that the presence of RRE (rev responsive element), the sequence within the mRNA in question to which rev binds, stimulates polymerization of rev under conditions in which the protein alone fails to assemble. These observations have prompted a model for the mechanism of action of rev in situ, whereby we envisage that RRE-nucleated polymerization of rev leads to a protective coating of the mRNA, protecting it from splicing enzymes and facilitating its export intact from the nucleus (Misra, Kocsis, Wingfield, Steven).

Multivariate Statistical Sequence Analysis of Intermediate Filament Rod Domains and Other Coiled-Coil Proteins.

We have been exploring the potentialities of multivariate statistical methods to analyze the α-helical rod domains of a data base of proteins. The 40 or so intermediate filament gene products have been grouped into six classes on the basis of tissue-specific expression and serology. We are attempting to evaluate how this classification squares with an objective, quantitative, classification based on a factorial analysis of the amino-acid sequences of their rod domains. Coiled coil sequences are characterized by multiple heptads (a-b-c-d-e-f-g) of amino-acid residues with hydrophobic residues predominantly occupying sites a and d. We have also extended the analysis to include other coiled-coil proteins. In this analysis, we have represented each sequence in terms of heptad occupancy, thus allowing sequences of different lengths to be compared. The results to date yield a classification that is largely consistent with the conventional scheme, but is somewhat sensitive to the numbers of factors used in the analysis. In addition to rationalizing relationships among intermediate filament proteins, we would like to be able to predict whether a newly discovered rod domain is likely to form a two-stranded or a three stranded coiled-coil, and whether it would preferentially form homo-oligomers or hetero-oligomers. (Conway, Trus, Steven).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27002-13 LSBR

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Biology of Macromolecular Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI :	Alasdair C. Steven, Laboratory Chief,	LSBR, NIAMS
	Margaret E. Bisher, Microbiologist	LSBR, NIAMS
	Frank P. Booy, Visiting Scientist	LSBR, NIAMS
	James F. Conway, Visiting Fellow	LSBR, NIAMS
	Heather L. Greenstone, Biologist	LSBR, NIAMS
	Eva Kocsis, Visiting Fellow	LSBR, NIAMS
	Manoj Misra, Visiting Associate	LSBR, NIAMS
	Benes L. Trus, Guest Worker	LSBR, NIAMS

COOPERATING UNITS *(if any)* Computer Systems Lab, Div. Computer Res. & Tech., NIH; Lab. of Skin Biology, NIAMS (Dr P Steinert); Dept. of Biology, Brookhaven Nat'l. Lab., (Drs J Wall, J Hainfeld); others as noted.

LAB/BRANCH

Laboratory of Structural Biology Research

SECTION

Section on Structural Biology

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	6	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This Laboratory aims to elucidate the regulatory mechanisms that govern the folding of macromolecules and the assembly of supramolecular complexes, and underlie the synthesis of organelles, cells, and tissues. To this end, advanced methods of structural analysis based on electron microscopy and computer image processing are developed and applied. In the past year, we have applied cryo-electron microscopy and three-dimensional image reconstruction to purified capsids of herpes simplex virus, type 1, which had been biochemically depleted in vitro of certain components, reassociated with those components, or decorated with monoclonal antibodies. The results have led to a provisional localization of the four minor capsid proteins, and have implications for their likely functional roles. VP22 (40kDa) is located entirely within the shell of (precursor) B-capsids, but is eliminated when DNA is packaged: most likely, it is a scaffolding protein that is at least partly responsible for controlling the polymerization of approximately 900 copies of the major capsid protein - VP5 (148 kDa) - into a large (125nm), precisely defined, icosahedral shell. VP26 (12kDa) appears to make up the pentons, with 80-100 copies of this small protein present at each of the five-fold vertices: VP26 may be involved in controlling release of the packaged DNA. VP19 (55 kDa) and VP23 (36 kDa) appear to be the triplex proteins, forming heterotrimers at the three-fold sites on the outer surface of the icosahedral surface lattice. By analogy with comparable proteins of other viruses, they probably serve to stabilize the capsid shell by reinforcing the underlying array of hexamers of VP5.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-539

Project Description:

Statement of Progress:
A. Development of Experimental Facilities:
(i) Computer Image Processing. We have completely renovated and commissioned a special-purpose image processing laboratory in Room B2 22 (Bldg 6), providing two high performance work stations, a sequestered space to which the bulk of the hardware has been consigned (thus reducing extraneous noise and heat), and a small amount of office space. Integral to this move have been an upgrade of our Microvax II to a Vax 4000-200, and acquisition of a Gould 9000 Image Processor. The latter is capable of supporting two work stations at 1024 x 1024 resolution, as compared to one station at 512 x 512 by its predecessor. Although additional work needs to be done in climate control, hardware acquisition (camera stations), and software adaptation, this laboratory is already operational and represents a great improvement in our experimental capability.
(ii) (Collaboration with C. Johnson, R. Martino, CSL/DCRT). As a part of our long term goal of performing three-dimensional reconstructions of large viruses at 1.0-1.5nm resolution, we have to improve our computing power by at least two orders of magnitude. As a potential solution to this problem, our software is being converted to run on the Intel 860s massively parallel computer that has recently been acquired by DCRT. This conversion is quite complex, requiring that the algorithms be redesigned to fully exploit the advantages of the parallel architecture. So far, one of the most time-consuming steps in the reconstruction has been successfully ported to the Intel, where it runs approximately 300-fold faster than on the VAX 8350 used in our earlier work.
(iii) Electron Microscopy. We have carefully formulated the specifications for a new high performance electron microscope, optimized for recording high resolution information from frozen, hydrated, specimens. We have also evaluated all potential commercial sources. Our specifications require non-standard (but attainable) modifications of the optical system, specimen stage, and image recording systems. Procurement is proceeding.

B. Structure and Assembly of Viruses.
(i) Capsid of Herpes Simplex Virus, Type 1 (collaboration with W Newcomb, J. Brown, University of Virginia Health Science Center). Our long term objective is to work out the assembly pathway of this archetypal member of an extensive family of animal viruses, several of which have important associations with human diseases. We have found that treatment of purified capsids with 2.5M guanidine hydrochloride quantitatively removes VP26. the smallest capsid protein, and causes the penton capsomers to disappear. Upon removal of the denaturant by dialysis, VP26 reassociates with the capsid, and material reappears at the vertex sites, albeit with an altered morphology compared with the original pentons. Exposure of capsids to a monoclonal antibody against VP5 results in strong labelling of the hexons but no labelling of pentons. The epitope has been localized with high precision to the extreme distal tip of the hexons' external protrusions. These data imply that pentons are composed of some protein other than VP5, and are thus consistent with the tentative identification of VP26 as the penton protein on the basis of the extraction experiments. Guanidine also releases a fraction of both VP19 and VP23, and causes the disappearance of most of the 120 peripentonal triplexes (there are 300 triplexes in all). Taking into account stoichiometry data, these observations tentatively suggest that the triplexes are heterotrimers consisting of one copy of VP19 and two copies of VP23. Our continuing experiments are aimed at testing and further refining this overall scenario by conducting similar experiments with other antibodies.

(ii) Maturation-dependent Conformational Change of Bacteriophage T4 Capsid. (Collaboration with P Ross, Laboratory of Molecular Biology, NIDDK; L Black, University of Maryland School of Medicine). We have continued our multi-disciplinary approach to elucidation of this exceptionally large-scale, irreversible, regulatory conformational change. Two phases of this work have been completed over the past year. In the first, monospecific antibodies were raised against four synthetic peptides that correspond to defined segments of gp23, the major capsid protein. These antibodies were used to label the precursor (uncleaved, unexpanded) and mature (cleaved, expanded) states of the capsid surface lattice and examined by immuno electron microscopy. The results demonstrate that at least three distinct peptides are translocated between the inner and the outer surfaces when expansion occurs. In the second study, differential scanning calorimetry was used to characterize the thermodynamic properties of two states that are intermediate between the precursor and the mature forms, viz. the cleaved unexpanded state (which occurs on the in vivo pathway), and the uncleaved, expanded state (which can be induced in vitro). The results provide a rationale for the cleavage reaction in that they demonstrate that a substantially greater stabilization accompanies expansion if it is preceded by full cleavage of gp23 to gp23*.

C. Other Projects.
In addition, progress has been made on investigations of (1) polymerization of the rev protein of human immunodeficiency virus: (2) three dimensional reconstruction of the crystalline porin of Bordetella pertussis: (3) analysis of the molecular structure of clathrin; (4) icosahedral reconstruction of the capsid of bacteriophage T7; (5) multivariate statistical analysis of intermediate filament protein sequences.

Publications

Booy FP, Newcomb WW, Trus BL, Brown JC, Baker TS, Steven AC. Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus, Cell 1991;64:1007-1015.

Steven AC, Bisher ME, Roop DR, Steinert PM. Biosynthetic pathways of filaggrin and loricrin - two major proteins expressed by terminally differentiated epidermal keratinocytes, J Struc Biol 1990;104:150-162.

Thomas L, Kocsis E, Colombini M, Erbe E, Trus BL, Steven AC. Surface topography and molecular stoichiometry of the mitochondrial channel, VDAC, in crystalline arrays, J Struc Biol 1991;106:161-171.

Mehrel T, Hohl D, Rothnagel JA, Longley MA, Bundman D, Cheng C, Lichti U, Bisher ME, Steven AC, Steinert PM, Yuspa SH, Roop DR. Identification of a major keratinocyte cell envelope protein, loricrin, Cell 1990;61:1103-1112.

Baker TS, Newcomb WW, Booy FP, Brown JC, Steven AC. Three-dimensional structures of maturable and abortive capsids of equine herpesvirus 1 from cryoelectron microscopy, J Virol 1990;64:563-573.

Steven AC, Greenstone H, Bauer AC, Bisher ME, Robey FA, Black LW. The maturation-dependent conformational change of phage T4 capsid involves the translocation of specific epitopes between the inner and the outer capsid surfaces, J Struc Biol 1991;106:221-236.

Fraser RDB, Furlong DB, Trus BL, Nibert ML, Fields BN, Steven AC. Molecular structure of the cell-attachment protein of reovirus: correlation of computer-processed electron micrographs with sequence-based predictions, J Virol 1990;64:2990-3000.

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Steinert PM, Mack JW, Korge BP, Gan S-Q, Haynes SR, Steven AC. Glycine loops in proteins: their occurrence in certain intermediate filament chains, loricrins and single-stranded RNA binding proteins. Int J Biol Macromol 1991;13:130-139.

Steven AC, Kocsis E, Unser M, Trus BL. Spatial disorders and computational cures, Int J Biol Macromol 1991;13:174-180.

Kocsis E, Trus BL, Steer CJ, Bisher ME, Steven AC. Image averaging of flexible fibrous macromolecules: the clathrin triskelion has an elastic proximal segment, J Struc Biol 1991 in press.

Wingfield PT, Stahl SJ, Payton MA, Graber P, Venkatesan S, Steven AC. HIV-1 rev expressed in recombinant Escherichia coli: purification, polymerization and conformational properties, Biochem 1991 in press.

Annual Report
Arthritis and Rheumatism Branch
Period Covered October 1, 1990 to September 30, 1991

I Disease Related Studies

A. Polymyositis

1. Etiology and Pathogenesis (ZO1AR 41074*)

Dr. Plotz and his colleagues are continuing integrated laboratory and clinical studies on polymyositis --a family of inflammatory diseases in which disease-specific autoantibodies occur and for which there is considerable indirect evidence pointing to a viral etiology. During the past year they have continued to collect blood and muscle specimens from patients as well as epidemiologic data. They have completed a search for potential etiologic viral agents using a highly sensitive polymerase chain reaction and failed to obtain positive results. They have extended their characterization of autoantibodies and shown that antibodies to tRNA can often be found at very early in the disease. A search for correlations between disease manifestations and specific HLA types is being extended. In order to pursue investigations of the role of histidyl-tRNA synthetase --the antigen against which many patients with this disease form autoantibodies--they have cloned, sequenced and expressed this enzyme. (A sequence of this protein published by others proved to be incorrect.) Control regions are also being analyzed. This work will give them the tools to try to unravel the relationship between the enzyme and the antibodies to it, and the disease.

2. Diagnosis(ZO1AR-41080)

Studies on the use of magnetic resonance imaging in the diagnosis and management of myositis were completed. Profound abnormalities of muscle phosphate metabolism were observed in patients with inclusion body myositis and in tryptophan-induced eosinophilia-myalgia syndrome, and lesser abnormalities in those with polymyositis and dermato-myositis. These studies have demonstrated that resonance imaging complements other techniques for evaluating disease activity particularly in research patients.

3. Treatment(ZO1 AR 41076)

Therapeutic trials on patients resistant to corticosteroid treatment are being pursued and involve, alternatively, plasma- and leukopheresis, intravenous methotrexate with leucovorin rescue, combination methotrexate and azathioprine, and i.v. cyclophosphamide. Analysis of data is underway. Poor results with some regimens led to discontinuance

in certain groups of patients.

B. Rheumatoid Arthritis

1. Animal Studies(ZO1AR 41048)

Streptococcal cell wall-induced arthritis in LEW/N rats strongly resembles rheumatoid arthritis in rats. Dr. Wilder's group continues to study this model. New studies have shown a role of cyclooxygenase in the arthritic process. In addition locally produced corticotropin releasing hormone and uteroglobin have been implicated in the pathogenesis.

Studies on the role of the hypothalamic-pituitary-adrenal(HPA) axis in susceptible and resistant rat strains are continuing. Recent results show strong correlations with certain behavioral characteristics consistent with differences in the HPA axis.

The differences in the rat strains appear to be assignable to a small number of autosomal genes and linkage studies have been initiated as a first step towards identifying the genes.

2.Patient studies(ZO1 AR 41066; ZO1AR 41088)

The observations in animals are being studied for their potential relevance in humans with rheumatoid arthritis. During the past year the study of synovial tissues have provided additional evidence for pathogenetic roles of platelet-derived growth factor, and heparin-binding fibroblast growth factor. The role of neuropeptides such as corticosteroid releasing hormone have been implicated in the human disease also as have cyclooxygenase and uteroglobin. All of these factors are only minimally expressed in patients with osteoarthritis.

Studies on the role of the HPA axis are also being pursued in human. RA patients were compared to appropriately matched controls with respect the activity of their HPA axis. The results are consistent with but do not prove a relationship between blunted responsiveness of the HPA axis and disease.

C. Systemic lupus erythematosus

1. Animal Studies(ZO1AR 41020)

Dr. Steinberg and his colleagues continue their studies of autoimmune mice in a long-term effort to determine the etiology and pathogenetic mechanisms of the human-like autoimmune diseases to which these animal are susceptible. Recent studies have focussed on the pluripotential stem cells and have demonstrated a fundamental abnormality is already detectable in the primitive cells from susceptible strains.

The role of retroviruses is also being pursued. Excess Mpmv RNA is found in the thymuses of disease-prone animals and this can be transferred with bone marrow stem cells. The genetic basis is being explored and there is evidence for defects in a negative regulatory region.

2. Human Studies

a)Pathogenesis(ZO1AR 41023)

T lymphocytes from patients appear to have an increase rate of mutation. Whether this is related to the disease process or results from prior treatment is currently under study.

Expression of endogenous retroviruses in human lupus and other human autoimmune disorders is continuing to be assessed. None of the retroviral probes have as yet demonstrated differences between the expression in T cells from normal vs affected individuals.

b)Treatment(ZO1 AR 41040)

The efficacy of various treatment regimens for preventing disease progression --particularly nephritis--continue to be tested. The three regimens currently being explored have failed to result in statistically significant differences during the first 2 years of follow-up.

D. Familial Mediterranean Fever (Z01 AR 41083)

This rheumatic disease is inherited as a single gene, autosomal recessive disorder. Dr. Kastner and his colleagues are attempting to identify the responsible gene. In turn this will permit one to probe the still-unknown biochemical basis of the disease.

Their analysis has so far excluded about 1/3 of the human genome, and they have strong indications that a, if not the, gene resides on the long arm of chromosome 17. Their data are beginning to demonstrate that different loci are likely to be involved in different families.

Strategies for progressively "homing in" on the gene(s) are being pursued.

II Basic Studies

Dr. Metzger and his colleagues continue their studies on the cell surface receptor with high affinity for IgE. This receptor plays a central role in allergic responses and serves as a highly useful model for studying activation of cells by ligand-induced aggregation of cell surface receptors.

During the past year this group has continued its study of genetically engineered mutant receptors transfected into cells and analyzed for various functions. Both the topological properties as well as

a variety of early biochemical perturbations have been assessed in transiently transfected COS cells and stably transfected P815 mastocytoma cells.

During the past year the aggregation-induced association of receptors with the detergent-resistant plasma membrane skeleton has been studied. The surprising finding is that none of the five cytoplasmic domains of the receptor are critical required to manifest this phenomenon. Most striking is that even the lipid anchored ectodomains of the alpha subunit demonstrate this effect. This suggests that the basis of this phenomenon may involve non-specific physical interactions rather than site-specific interactions as had been supposed.

A major effort has been directed towards developing stable transfectants bearing mutant receptors in cells that exhibit some of the early events associated with transmembrane signalling by wild-type receptors. Such cells have now been analyzed and they implicate both the cytoplasmic extension of the gamma chains and the carboxy-terminal cytoplasmic extension of the beta chain as being critical. The molecular details of how these regions are involved in signal transduction are being pursued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41020-24 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathogenesis of Autoimmunity in Mice With SLE-like Illness

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
P.I. Alfred D. Steinberg, Chief, Cellular Immunology Section, ARB,NIAMS
Mark Gourley, Special Volunteer, Cellular Immunology Section, ARB,NIAMS
Leslie King, IRTA Fellow, Cellular Immunology Section, ARB,NIAMS
Wendy Kisch, Biologist, Cellular Immunology Section, ARB,NIAMS
Arthur M. Krieg, Special Volunteer, Cellular Immunology Section, ARB,NIAMS
Dorothy Scott, Special Volunteer, Cellular Immunology Section, ARB,NIAMS
William Schweiterman, Senior Staff Fellow, Cellular Immunology Section, ARB,NIAMS
Geryl Wood, Biologist, Cellular Immunology Section, ARB,NIAMS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIAMS - Building 10, Room 9N218, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In lupus-prone strains of mice, bone marrow derived pluripotent stem cells and/or their immediate progeny demonstrate excessive proliferation. They have increased numbers of both endogenous and exogenous stem cell derived colonies. In vitro studies with NZB marrow demonstrate an excess of stem cell derived B cell production of IgM and anti-DNA relative to other strains. The results were confirmed and extended using sorter purified stem cells. In addition, such sorter purified stem cells transfer the NZB phenotype to normal mice. These studies suggest a fundamental abnormality in stem cell activity.

Retrovirus studies demonstrate excess Mpmv RNA in thymuses of lupus-prone mice. The abnormality is transferrable with bone marrow stem cells. The basis for this abnormal expression has been studied. First, a genomic library from NZB mice was probed with Mpmv specific probes and a full-length clone isolated. The LTR was sequenced, demonstrating a mutation in a negative regulatory region. Gel retardation studies confirm that the mutation alters binding of a regulatory DNA binding protein.

Project Description:

Objectives

Several strains of mice spontaneously develop autoimmune diseases resembling human systemic lupus erythematosus. Early in life they are immunologically hyperactive, become relatively resistant to tolerance induction, and gradually develop antinuclear and other autoantibodies followed by clinical disease. We believe that a detailed understanding of the genetic, cellular and biochemical basis for disease in these mice will contribute to the understanding of a variety of immune mediated diseases.

Methods Employed

1. Studies of peripheral lymphoid cells: Assays for antibodies to RNA, DNA and other ligands using the ammonium sulfate precipitation or the ELISA technique. Assay of antibody forming cells to a number of antigens by a modified ELISA assay. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants. Studies of $CD5^+$ B cells, including peritoneal cells. Transfer of spleen, bone marrow, peritoneal, and thymus cells into recipients to evaluate the functional properties of the different cell types. Separation of lymphoid cells using monoclonal antibodies and either C-mediated cytotoxicity or magnetic beads. If greater purity is needed, the cells are further separated on the cell sorter. Evaluation of subpopulations of cells using the fluorescence activated cell sorter.

2. In vitro culture of marrow cells by two different methods: Whitlock-Witte (which favors the emergence of lymphocytes) and Dexter (which does not).

3. Studies of thymocyte differentiation in vitro using a variety of cytokines to stimulate either proliferation or differentiation. Special emphasis is placed on $CD4^-$, $CD8^-$ thymocytes, the precursors to the other cells. Normal thymocytes from fetal and adult animals are studied prior to studies of autoimmune animals.

4. Molecular studies of RNAs: Northern blot analyses. Isolation of poly A^+ mRNA from lymphoid organs or cell subsets or cell clones and probing. Nuclear run-off to determine whether increased gene expression is due to increased specific mRNA production. In situ hybridization using riboprobes. Separate isolation of nuclear and cytoplasmic RNA to determine partition of different messages.

5. Southern blot analyses.

6. Culture of spleen cells with deoxyribonucleotides anti-sense to known retroviral sequences. In vivo administration of anti-sense oligonucleotides. We use phosphorothioate oligonucleotides for these studies since they are much more resistant to nucleases than are standard oxygen containing oligos.

7. Probing of genomic and cDNA libraries and isolation of relevant clones. Gene cloning and sequencing using standard techniques.

8. Transfection of genes into cell lines.

9. Production of transgenic mice.

Major Findings

1. We have previously found that lupus -prone mouse strains, NZB, BXSB, MRL-+/+, MRL-lpr/lpr, and (NZB x NZW) F1, all have full-length, 8.4 kb, Mpmv retroviral transcripts in their thymuses whereas normal mice do not. The studies suggested that Mpmv 8.4 kb mRNA represents the action of a gene associated with autoimmunity and does not result from disease itself. We now have taken bone marrow stem cells from lupus-prone NZB or BXSB mice and transferred them to normal histocompatible, lethally irradiated, DBA/2 and C57BL/6 recipients, respectively. The reciprocal transfer also was done. The resulting Mpmv expression in the thymus was determined by the donor marrow. Therefore, the marrow, rather than thymic epithelial cells or other host factors, determines the abnormal lupus-specific gene expression.

2. Several clones were obtained from an NZB genomic library using an Mpmv-specific probe and others using a Pmv-specific probe. The LTRs of full length and less than full-length clones were sequenced and compared with the known sequences from non-lupus prone mice. NZB Mpmv LTRs were found to contain a mutation in a negative regulatory region. Gel retardation assays were performed using DNA binding proteins which bind to that region. There was less inhibition by the mutant NZB LTR sequence than by the wild type. That study suggests that the mutation does reduce binding of the DNA binding proteins. Studies in progress are directed at transfecting (transient transfections) mutant and wild-type sequences in a CAT assay system to determine if there is a functional difference.

3. We previously have shown in vitro uptake by spleen cells of oligonucleotides anti-sense to the initiation region of the Mpmv env gene. The effect was sequence specific stimulation of lymphocyte activation. It did not occur in rat cells which lack Mpmv endogenous sequences. Phosphorothioate oligonucleotides, resistent to nucleases, are much more effective than oxy-oligos. We believe that the stimulation results from release of the cells from inhibition by an env gene product. We have now administered the phosphorothioate olgionucleotides in vivo. Those results demonstrate in vivo lymphocyte stimulation by anti-sense analogous to the prior in vitro stimulation. There was no stimulation by a scrambled control oligo in vivo. Moreover, the in vivo studies showed a dose-dependent stimulation with doses from 1.5 ug per mouse to 1,500 ug per mouse. With maximal in vivo stimulation, there was no further stimulation when spleen cells were cultured in vitro with anti-sense after the in vivo treatment. The in vivo studies demonstrated a dose-dependent stimulation of thymocyte

DNA synthesis by anti-sense when thymocytes were pulsed immediately after harvest and assayed 4 hours later. It previously was not possible to do a comparable study in vitro since the thymocytes had to be cultured for a substantial period of time with anti-sense prior to pulsing.

4. Bone marrow from lupus-prone strains was found to have increased stem cell activity when compared to normal mice. This was true using both the endogenous colony forming system and the exogenous colony forming system. Bone marrow from NZB and BXSB mice was sorted to remove cells already down the differentiation path toward one marrow product or another and, thereby, purify stem cells (Thy 1^{lo} Lineage $marker^{neg}$). These sorter purified stem cells were used to reconstitute histocompatible normal recipients which had been lethally irradiated. Mice survived with as few as 100 injected cells. Recipients of NZB sorter purified stem cells developed autoimmune features: increased colony forming units, anti-DNA, hypergammaglobulinemia.

5. Bone marrow from lupus-prone and normal mice was grown in vitro under two different conditions, one which favors the emergence of lymphocytes and the other which does not. Fewer seeded stem cells from NZB marrow gave rise to lymphocytes in Whitlock-Witte cultures. There also were increased numbers of B cells as well as increased immunoglobulin production. Preliminary studies suggest that there is not an increase in granulocytes and macrophages. NZB mice may have a preferential skewing of stem cell progeny towards B cell production. Sorter purified stem cells were able to grow in long-term Whitlock-Witte cultures on NZB and DBA/2 and Balb/c stroma.

6. Sorter purified stem cells from NZB mice with the phenotype $Thy1^{lo}$ Lineage $marker^{negative}$ (Lin^{neg}) gave rise to normal numbers of stem cell colonies. In contrast, sorted stem cells with the phenotype Thy 1^{neg} Lin^{neg} led to a marked increase in stem cell colonies (1_5 per 10^2 seeded cells, whereas normal mice have $< 1/10^5$). Thus, Thy 1^{neg} Lin^{neg} stem cells may be a separate lineage of abnormal stem cells in NZB mice.

7. Autoimmune-prone and normal mice were given staphylococcal enterotoxin B (SEB) intravenously. At times up to three weeks later, the mice were studied for numbers of T cells bearing different beta chains of the T cell receptor. In addition, their ability to be stimulated in vitro by SEB was assessed. (NZB x NZW) F_1 and MRL-lpr/lpr mice showed a quantitative reduction in deletion of Vß8 T cells reactive with the SEB. The

remaining cells were not tolerant, but responded more than cells from control strains to restimulation with SEB. These stuides demonstrate a defect in peripheral tolerance of two kinds, and allow us to further dissect the basis of abnormal tolerance in lupus-prone mice.

Significance to Bio-medical Research and the Program of the Institute

The diseases of mice occur spontaneously and are a close approximation of the comparable human diseases. The cellular and genetic bases of disease increasingly are becoming better understood. Such insights provide a firm basis for the investigation of the human diseases as well as strategies for immune intervention.

The tolerance studies are directed at the defect immediately proximal to the production of autoimmune responses. They demonstrate at least two defects in peripheral tolerance in lupus-prone mice.

Expression of a unique retroviral transcript in association with murine lupus provides a possible clue to early events in, or even pathogenesis of, murine lupus. In addition, evidence that endogenous retroviral sequences may play a role in normal lymphocyte activation provides a possible pathway for abnormal lymphocyte activation in autoimmune diseases.

Since the information for murine lupus is contained in the bone marrow stem cell, that cell could be a mere conduit of the information or could, itself, be phenotypically abnormal. Studies demonstrating abnormalities of the stem cells may provide a better understanding of lupus and could lead to more directed treatments.

Proposed Course

The role of retroviral expression will be further dissected in normal and autoimmune-prone mice. RNA turnover studies should better define the abnormal retroviral gene expression observed in autoimmune thymuses. The clones we have obtained will be further studied in transfection experiments and we hope to produce transgenic mice containing them. Similarly, we hope to move the anti-sense studies to the transgenic model.

We wish to continue to dissect the bone marrow stem cell and its possible gene dysregulation in murine lupus. Studies are underway to assess stem cell activity in patients with systemic lupus.

Publications:

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2. Steinberg, A.D. Tolerance and autoimmunity, Chapter 229 (pp 1655-1658) in Internal Medicine, edited by Jay H. Stein, Little Brown and Company, Boston, 1990.

3. Steinberg, A.D. Mechanisms of Disordered Immune Regulation, in Basic and Clinical Immunology, Seventh Edition, ed. D.P. Stites and A.I. Terr, Appleton & Lange, Norwalk, CT, 1991, pp. 432-437.

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6. Takashi, T., Gause, W.C., Wilkinson, M., MacLeod, C.L., and Steinberg, A.D. IL 1-induced differentiation of progenitor thymocytes. Europ. J. Immunol. 21: 1385-1390, 1991.

7. Krieg, A.M., Gmelig-Meyling, F., Gourley, M.F., Kisch, W.J., Chrisey, L.A. and Steinberg, A.D. Uptake of oligodeoxy-ribonucleotides by lymphoid cells is heterogeneous and inducible. Antisense Research and Development 1991 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41022-19 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Therapeutic Studies in Murine Lupus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Alfred D. Steinberg, Chief, Cellular Immunology Section, ARB,NIAMS

INACTIVE DURING THIS PERIOD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Arthritis and Rheumatism

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NIAMS - Building 10, Room 9N218, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type Do not exceed the space provided)*

Several mouse strains and crosses spontaneously develop autoimmune immune-mediated pathology resembling that of human systemic lupus and other rheumatic autoimmune diseases, including Sjogren's syndrome and rheumatoid arthritis. (NZB x NZW)F_1 mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb's positive hemolytic anemia, membranous nephrosis, lymphadenopathy, antibodies to DNA and glomerulonephritis. MRL-lpr/lpr mice develop massive lymphadenopathy, arthritis, arteritis, nephritis and produce very large amounts of IgG antibodies. BXSB mice and their hybrids with NZW and NZB mice develop degenerative coronary artery disease in addition to glomerulonephritis. These mice are excellent models for studying different therapeutic programs. The overall objective is to develop potentially promising new therapies which ultimately might be beneficial in the treatment of patients.

Comparison of different therapeutic regimens on the natural history of mice with lupus by study of autoantibodies, proteinuria, renal and coronary pathology, lymphadenopathy, and survival.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Major Findings

Proposed course:

Ultimately we would like to extend therapeutic mouse studies to humans; however, the agents so far tested are not of sufficient promise to supplant currently used human therapies. We are trying to obtain more useful reagents for that purpose.

Publications:

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41023-17 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 charactars or less Title must fit on one line between the borders.)*
Studies of Patients with Immune-mediated Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
P.I. Alfred D. Steinberg, Chief, Cellular Immunology Section, ARB, NIAMS
Sahar Dawisha, Clinical Associate, Cellular Immunology Section, ARB, NIAMS
Fritz Gmelig-Meyling, Visiting Scientist, Cellular Immunology Section, ARB, NIAMS

Collaborative: Dr. Dennis Klinman, FDA

COOPERATING UNITS *(if any)*

LAB/BRANCH
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SECTION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.6	0.2

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with systemic lupus were found to have an increase in mutations in their T cells as defined by their ability to grow in the presence of 6-thioguanine. The extent to which this results from a primary abnormality and the extent to which drugs contribute remains to be determined.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description:

Objectives

To understand the pathogenesis of human immune mediated diseases. This will include analyses of genetic, immune, and environmental factors which might contribute to disease. Special emphasis will be placed upon genetic factors. We will try to capitalize upon advances in murine studies.

Methods Employed

1. Study of patients and families with SLE, polymyositis, rheumatoid arthritis, and angioimmunoblastic lymphadenopathy with regard to immune functions.

2. Isolate T cells from patient sample. Grow T cells in vitro with and without 6-thioguanine. Cells with a normal hprt gene make hypoxanthineguanine phosphoribosyl transferase which converts the 6-TG to toxic substances. Cells with a mutation in this gene may no longer die when grown in the presence of 6-TG. The number of cells grown at limiting dilution able to grow in 6-TG gives a measure of the spontaneous mutation frequency in the T cells.

3. After clonal expansion, T cells are stimulated in vitro with various self-antigens to measure specific stimulation.

4. Study antibody forming cells from patients with systemic lupus by an ELISA spot assay.

5. Northern analyses of endogenous retroviral expression.

6. Map and then clone gene(s) associated with disease.

Major Findings.

1. Several patients with systemic lupus have increased numbers of T cells able to grow in the presence of 6-thioguanine relative to normal controls. The mutation frequency in normals was approximately 5×10^{-6}. That in patients varied from an increase of 3 - 100 fold. We are in the process of determining the magnitude of this effect in patients who have received various therapies to determine the extent to which it is a spontaneous abnormality and the extent to which drugs may have contributed.

2. T cell clones from patients with systemic lupus are stimulated by self-antigens in vitro. This observation requires additional studies for definition of the magnitude and specificity of the observation.

3. Patients with lupus have an increase in numbers of antibody forming cells of many specificities indicating that they have polyclonal B cell activation. They have a disproportionate increase in cells making antibodies reactive with DNA, indicative of a specific stimulation. Both processes occur simultaneously in the same patient.

4. We have previously found that peripheral blood cells from patients with several autoimmune rheumatic diseases were found to contain endogenous human retroviral RNA. Such RNA was also found in some normal individuals. Using probes not available last year we continue to find expression of additional families of retroviral sequences, but no differences between patients and controls in human endogenous retroviral expression in peripheral blood cells.

Significance to Bio-medical Research and the Program of the Institute

We are trying to establish the basis of diseases at a molecular level and determine a possible genetic basis of diseases. The immune abnormalities may be important in disease pathogenesis, and may be a guide to therapy, but do not define a specific molecular defect.

Proposed Course

Define the mutation frequency in patient T cells independent of drug effects and then determine the drug effects, if any. Study the nature of the mutation abnormalities. Determine the magnitude and specificity of T cell responsiveness to self-antigens.

Continue to obtain new human endogenous retroviral probes as they become available to determine if any define a uniquely expressed species in an autoimmune rheumatic disease.

Publications:

1. Klinman, D.M. and Steinberg, A.D. Antibody forming cells in patients with systemic lupus erythematosus. Arthritis Rhem. 1991 (in press)

2. Steinberg, A.D., Gourley, M.F., Klinman, D.M., Tsokos, G.C., Scott, D.E., and Krieg, A.M. Pathogenesis of systemic lupus erythematosus. [edited transcript of Combined Staff Conference, Feb. 27, 1991] Ann. Intern. Med. 1991 (in press).

3. Steinberg, A.D. Systemic lupus erythematosus, in Cecil Textbook of Medicine, Nineteenth edition, ed. J.B. Wyngaarden, L.H. Smith, Jr., J.C. Bennett, & F. Plum, W.B. Saunders Co., 1991 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41025-20 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the cell surface receptor for IgE

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	H. Metzger	Chief, Sec. Chem. Immunol	ARB/NIAMS
	S. Mao	Visiting Associate	ARB/NIAMS
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COOPERATING UNITS *(if any)*
none

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Chemical Immunology Section

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9	8	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The high affinity receptor for IgE on mast cells and basophils plays a central role in immediate hypersensitivity reactions. Crosslinking of bound IgE by polyvalent antigen leads to aggregation of the receptors and cellular secretion of both preformed and newly synthesized mediators of inflammation. The molecular mechanisms by which aggregation of the receptors generates cellular responses are still largely undefined. During the past year we have continued to employ mutated receptors in order to analyze which portions of the receptor participate in its principal functions. Our new results show: 1) None of the cytoplasmic domains are required for receptors to exhibit detergent-insolubility after aggregation. Indeed, lipid-anchored ectodomains of the alpha subunit are sufficient to demonstrate this effect. 2) A receptor-less mast cell-like line has been successfully transfected with various mutant receptors and studied with respect to the early biochemical events associated with aggregation of the wild-type receptors for IgE. Truncation of the cytoplasmic domain of the alpha subunit or at the amino-terminus of the beta subunit failed to inhibit signalling. Truncation of the carboxy-terminus of the beta caused a loss of function not only of the IgE receptors but also of the endogenous IgG receptors. This is interesting because the latter are not known to associate with beta chains. Truncation of the gamma chains also led to loss of functions. In this case the receptors for IgG were also inactivated possibly because the functionally active receptors are of the type that contain gamma subunits. Surprisingly the inactive IgE receptors were found to contain normal amounts of the wild type (endogenous) gamma chains. This suggests that the transfected mutant gamma chains had a negative dominant effect.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description:

Objectives:

IgE is a unique class of immunoglobulin that plays a predominant role in the pathophysiologic events associated with a variety of allergic phenomena. The IgE mediates its functions by binding to plasma membrane receptors on mast cells and related cells. The binding itself is not known to produce changes; however, if the cell bound IgE becomes aggregated (e.g. by binding to a multivalent antigen), rapid degranulation of the cell ensues. It is now known that the critical initiating event is aggregation of the receptor to which the IgE is bound. Our objective is to understand the immediate molecular sequelae of this aggregation since this may have implications for therapeutic manipulation of this system as well as of other antibody-mediated cell perturbations. Our principal goal is to discover so-called "post receptor" molecules (PRM) with which the aggregated IgE receptors interact following aggregation. Our principal tactic is to use mutational modification of the receptor in order to identify those portions of the receptor that mediate the interaction between the receptor and one or more PRM. Such regions of the receptor could then be used as probes to study the receptor-PRM interactions, to attempt to inhibit the interactions, and to isolate the PRM.

Methods Employed

The principal new method employed during this year's work was fluorescence activated cell sorting both for analysis and for preparative sorting and cloning.

Major Findings

1) Internalization of mutant receptors. We had previously observed that when IgE receptors ($Fc_\epsilon RI$) transfected into COS cells were aggregated they would internalize albeit much more sluggishly than the endogenous receptors on rat basophilic leukemia cells. When $Fc_\epsilon RI$ with truncated cytoplasmic domains (CD) were tested in COS cells, they behaved surprisingly like wild-type receptors. The one exception were receptors containing a beta chain with a truncated carboxy-terminal cytoplasmic domain. Nevertheless, receptors containing human alpha chains - which can be expressed when co-transfected with gamma chain alone i.e. without beta chains - internalized as well as wild-type receptors. It therefore appears that the beta chain does not contain a site that is absolutely critical for receptor internalization. We have extended those studies to the internalization of receptors transfected permanently into P815 cells. On these cells the wild-type receptors are internalized efficiently - as rapidly as they are in RBL cells. When mutated $Fc_\epsilon RI$ were transfected into P815 cells, they behaved just like they did on COS cells. We conclude that whatever the mechanism is by which the receptors are rapidly internalized after aggregation, it does not require interactions involving specific sites in the cytoplasmic domains on the $Fc_\epsilon RI$.

2) Studies on the interaction between $Fc_\epsilon RI$ and cell skeletal components. After aggregation, the $Fc_\epsilon RI$ remain associated with the cell

remnant after treatment of the cells with mild detergents. It has been assumed that this results from new interactions between the aggregated receptors and "cytoskeletal" components. More recently interaction with "plasma membrane skeleton" on the cytoplasmic side of the plasma membrane has been proposed. We have examined our mutant $Fc_{\varepsilon}RI$ both in transiently transfected COS cells as well as in permanently transfected P815 cells. Again, all the mutants behaved as wild-type receptors except for those containing a beta subunit with a truncated carboxy-terminal CD. Two additional molecules also behaved as the wild-type receptors: one, a chimeric protein containing the ectodomains of the $Fc_{\varepsilon}RI$ and the transmembrane and cytoplasmic domain of the p55 subunit of the IL-1 receptor (the so-called Tac protein); the other an alpha subunit genetically modified so that it is expressed as a lipid-anchored rather than a transmembrane protein. We conclude that whatever interactions account for the detergent insolubility of the aggregated receptors, they do not require specific sites on any of the CDs of the $Fc_{\varepsilon}RI$. Possibly such specific sites are on the ectodomains of the alpha chains.

3) Activation of transfected mast cells

a) Preparation of permanently transfected cells lines. Considerable effort was expended in order to get each type of mutated receptor into at least two cell lines by separate transfections, such that they were permanently expressing substantial numbers of receptors. The use of a panning procedure, fluorescent activated cell sorting (and cloning) and screening of clones with a solid phase enzyme-linked assay proved to be a relatively reliable procedure for successfully isolating usable transfectants.

b) Functional assessment of clones. We have analyzed some of the early biochemical events triggered by transfected mutant receptors after aggregation. In addition to the rise in cytoplasmic Ca^{2+} and hydrolysis of phosphoinositides, we also assessed the phosphorylation of tyrosines on cellular proteins after stimulation. The three functional assays yielded concordant results in all the clones and mixed cell cultures tested. Each cell line was also tested for its responsiveness to thrombin. None of the cells transfected with mutant $Fc_{\varepsilon}RI$ showed a reduced response to thrombin when compared to those transfected with intact $Fc_{\varepsilon}RI$.

Truncation of the cytoplasmic domain of the alpha subunit or at the amino-terminus of the beta subunit failed to inhibit signalling. Truncation of the carboxy-terminus of the beta caused a loss of function not only of the IgE receptors but also of the endogenous IgG receptors. This is interesting because the latter are not known to associate with beta chains. Truncation of the gamma chains also led to loss of function. In this case the receptors for IgG were also inactivated possibly because the functionally active receptors are of the type that contain gamma subunits. Surprisingly the inactive IgE receptors were found to contain normal amounts of the wild-type (endogenous) gamma chains. This suggests that the transfected mutant gamma chains had a negative dominant effect.

4) Summary of CRADA Activity

Collaborative efforts were continued under CRADA OTT Ref. 89/402 with HOFFMANN-LAROCHE, INC. This CRADA which was initiated in October 1989 is currently funded at $30,000 p.a. to support postdoctoral fellows. The objectives of the CRADA are to gain insight into how the high affinity receptor for IgE mediates its two functions: binding IgE and initiating cellular degranulation in response to aggregation of the receptor. Such information can then form the basis for developing specific drugs to interfere with these two functions in order to prevent and/or counteract allergic reactions. During the past year there has been a close collaborative effort. This has involved frequent phone discussions to seek mutual advice, exchange of specific genetic constructs, cell lines, and antibodies. The work accomplished at the NIH is detailed in sections 1-3 and principally involves investigations of transmembrane signalling. The work accomplished at Hoffmann LaRoche has focussed heavily on the IgE binding and has included the use of antibodies to the IgE-binding domains of the receptor, IgE peptide sequences as inhibitors of IgE-binding and attempts to define the IgE-binding domain through genetic modifications of the alpha subunit of the receptor. In all of these studies, molecular genetic approaches have played the major, indeed preeminent, role. The principal observations raise doubts on the previously accepted data from another laboratory that the receptor binding site on IgE can be mimicked by a 76 amino acid peptide from the CH2:CH3 interface of the Fc region of IgE. In addition the Collaborators have genetically constructed chimeric proteins to probe structural questions and have developed permanently transfected cell lines that can be stimulated via the human IgE-binding subunit. The latter will be an important tool in screening for inhibitors of human IgE binding.

Significance to Biomedical Research

Mutational analysis is providing a powerful tool with which to identify regions of the IgE receptor that are critical for a variety of cellular responses that the receptor initiates. Although so far we have principally identified regions that are not essential for the functions being examined, such results are in fact the most easily interpreted. (When a mutation leads to a loss of function, there is always the concern that the effect is not due to the direct involvement of the region in question, but results instead from an indirect effect on the receptor's structure.) Once critical regions are defined, this information can be used to try to identify critical "post-receptor" macromolecules with which the receptor interacts. Interfering with such interactions can then be studied as a means by which to interfere with receptor function in allergic reactions.

Proposed Course

1) Studies on receptor movements. We are beginning studies in collaboration with Dr. Janet Oliver to determine the mechanism by which the receptors on transfected cells are internalized. The surprising

result that the cytoplasmic domains appear not to play a significant role is contrary to the results obtained with related Fc receptors whose endocytosis via a coated pit mechanism appears to involve the cytolasmic domains.

We also plan to study receptors in which one or more of the subunits have been modified ("flagged") such that they contain a cell surface-exposed epitope by which the receptors can be aggregated with antibodies specific to that epitope. This will permit us to probe more directly whether the phenomena we are observing are related to specific interactions between critical sites on the receptor or results from aggregation per se.

2) Studies on functional transfectants. We plan to investigate the mechanism by which certain mutations of the beta and gamma subunits lead to inactivation. The availability of plasmids for the Fc_γ receptors and for "flagged" subunits of the Fc_ε receptors should provide us with useful tools for such studies.

3) Studies on protein phosphorylation. Earlier studies by our own group as well as more recent work in other laboratories has pointed to the importance of protein phosphorylation as an early event in the signalling mechanism of the receptor. We have begun to explore the phosphorylation of various cellular substrates including the receptor itself, and the association of the receptors with protein kinases before and after aggregation.

Publications

Varin-Blank, N and Metzger H. Surface expression of mutated subunits of the high affinity mast cell receptor for IgE. J Biol Chem 1990;265:15685-94. (previously listed as in press).

Mao S-Y, Edidin M, Metzger H. Immobilization and internalization of mutated IgE receptors in transfected cells. J Immunol 1991;146:958-66.

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Metzger H. The high affinity receptor for IgE on mast cells. Clin Exp Allergy 1991;21;1-11.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41040-19 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Various Cytotoxic Drug Programs in Diffuse Lupus Nephritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
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Mark Gourley, Special Volunteer, Cellular Immunology Section, ARB,NIAMS
Dorothy Scott, Special Volunteer, Cellular Immunology Section, ARB,NIAMS
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COOPERATING UNITS *(if any)*
James E. Balow, Senior Investigator, NIDDK
Howard A. Austin, Attending Nephrologist, Clinical Center Foreign: NONE

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIAMS - Building 10, Room 9N218, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients were randomized to receive (i) monthly IV-cyclophosphamide, (ii) monthly IV methylprednisolone, or (iii) both. The study is now closed to new entrants. Rate of progression to renal failure, requirements for re-treatment, and drug toxicities are being assessed. Clear-cut differences have yet to emerge for the entire group of patients completing 2 years of study.

Project Description:

Objectives

The Arthritis and Rheumatism Branch has a major interest in the management of the nephritis of systemic lupus erythematosus (SLE). Renal failure is one of the most feared complications in the disease. It has been suggested that drugs capable of altering the antibody response, "immunosuppressives", would reduce the formation of antibody and thus of the immune initiation of renal inflammation. The objective of this study is to assess the efficacy and toxicity of various immunosuppressive drug programs in SLE nephritis. One particular goal is to determine if bolus methylprednisolone might be as efficacious as cyclophosphamide, but be less toxic.

Methods Employed

Patients with systemic lupus erythematosus and active nephritis have been entered into several trials. All patients are repeatedly reevaluated with a tests of renal inflammation and renal function, serological studies, and urinalyses. Treatment failures can be withdrawn according to protocol. Long-term follow-up of patients previously admitted continues. Our latest protocol involved randomizing patients to receive monthly one of the following: (i) bolus cyclophosphamide, (ii) bolus methylprednisolone, or (iii) both. Eighty patients have now been recruited and the study is closed to new patients.

Major findings

1. A two year follow-up of patients in the current study has not provided evidence for clear-cut differences between the groups.
   Renal failure has been observed in a few patients, including those randomized to receive cyclophosphamide. Re-treatment has been required for many patients in all three groups. The average changes in urine sediment, proteinuria, and serum creatinine are not different among the three groups at one year or at two years.

Significance to Bio-Medical Research and the Program of the Institute

These studies are relied upon by physicians concerned with SLE all over the world. We have been one of very few units able to speak from the hard data base of controlled studies.

Proposed Course

We will continue to follow these patients on their assigned programs.

Publications:

1. Steinberg, A.D., Kreig, A.M., Takashi, T., and Gourley, M.F. Timing of immunosuppression in the history of autoimmunity. Proceedings of the Second International Symposium on Immunomodulation, Paris, May 13-16, 1991.

2. Steinberg, A.D. and Steinberg, S.C. Long-term preservation of renal function in patients with lupus nephritis randomized to receive cyclophosphamide-containing regimens as compared with prednisone only. Arthritis Rheum. 1991 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT -

PROJECT NUMBER

Z01 AR 41048-12 ARB

RIOD COVERED

:tober 1, 1990 to September 30, 1991

LE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

acterial cell wall-induced arthritis and hepatic granuloma formation in the rat

NCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	R.L. Wilder	Sr. Investigator	ARB/NIAMS
	H. Sano	Visiting Fellow	ARB/NIAMS
	L. Crofford	Med. Staff Fellow	ARB/NIAMS
	E. Goldmuntz	Med. Staff Fellow	ARB/NIAMS
	J. Cash	Med. Staff Fellow	ARB/NIAMS
	E. Remmers	Sr. Staff Fellow	ARB/NIAMS
	K. Kalogeras	Sr. Staff Fellow	ARB/NIAMS

OPERATING UNITS *(if any)*

Clinical Neurosciences Branch, NIMH
Developmental Endocrinology Branch, NICHD

Holland Labs, American Red Cross

/BRANCH

Arthritis and Rheumatism Branch

CTION

Connective Tissue Diseases

TITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

AL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	

ECK APPROPRIATE BOX(ES)

(a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

MMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Streptococcal cell wall arthritis in LEW/N rats closely resembles rheumatoid arthritis in humans. New data have provided insights into the role of cyclooxygenase in the arthritic process. Moreover, locally produced corticotropin releasing hormone and uteroglobin have also been implicated in the disease process.

We have previously presented data indicating that a defective hypothalamic-pituitary-adrenal (HPA) axis response is associated with the extreme susceptibility to arthritis in LEW/N rats and relative resistance in F344/N rats. We have now extended our studies to show that LEW/N and F344/N rats show characteristic behavioral responses consistent with their blunted and robust HPA axis responses, respectively. Ontogenetic studies of the stress response have also shown profound differences between LEW/N and F344/N rats. These observations provide new insights into the role of the stress response and the development of human autoimmune diseases.

Acute streptococcal cell wall arthritis severity in LEW/N and F344/N rats is genetically controlled by a limited number of autosomal genes. Genetic linkage studies have been initiated to identify these genes.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864 939

Project Description:

Background and Objectives

Bacterial cell wall-induced arthritis is an experimental animal model of chronic proliferative and erosive synovitis, closely resembling rheumatoid arthritis, that is induced by systemic administration of biodegradation resistant bacterial cell wall fragments in aqueous solution. The development of disease is under the control of genetic factors. Previously published data from our laboratories have shown that arthritis-susceptible LEW/N rats have a profound defect in the HPA axis response to various inflammatory stressors, whereas arthritis-resistant F344/N rats have robust responses. Studies in progress have the following goals:

A. To characterize the host molecular genetic factors that regulate susceptibility to develop arthritis.

B. To better define pathophysiologic processes by characterizing differences between high and low responder inbred rat strains.

C. To define mechanisms leading to tissue injury and destruction.

D. To develop techniques to modulate the process.

Major New Findings:

1. Mechanisms of tissue injury:

   a. A study of the expression of cyclooxygenase *in situ* in arthritic joints is continuing. This enzyme is the rate-limiting factor in the synthesis of prostaglandins, which are believed to play an important role in inflammatory arthritis. Enhanced expression of both mRNA and protein has been demonstrated. Like other inflammatory mediators, expression is also thymus-dependent. It is minimal in arthritis-resistant F344/N rats. Corticosteroids are potent downregulators of this important enzyme.

   b. Uteroglobin is a potent phospholipase A2 inhibitor. We have been studying its expression in arthritic joints, as well as other tissues. Enhanced expression of both mRNA and protein has been clearly demonstrated in arthritic LEW/N rats. Its expression is minimal in F344/N rats. Additional *in vivo* and *in vitro* studies are in progress to understand the importance of these observations.

c. High level expression of corticotropin releasing hormone (CRH) has been demonstrated in arthritic joints of LEW/N but not F344/N rats. These, and other observations, suggest that CRH is an important autocrine/paracrine proinflammatory regulator of inflammation. These observations are particularly interesting because hypothalamic production of CRH in LEW/N rats is defective but is robust in F344/N rats. These observations provide new insights into immune system-central nervous system interactions.

2. Studies of neuroendocrine regulatory mechanisms in LEW/N and F344/N rats:

   a. LEW/N rats, compared to F344/N rats, have blunted behavorial responses to a wide variety of environmental stimuli, e.g., new environment, swim stress, restraint, ether. These responses are strongly associated with blunted and hyperactive HPA axis responses in LEW/N and F344/N rats, respectively.

   b. Studies of the ontogeny of the HPA defect have continued. Data indicate that HPA axis responses are blunted throughout the lifespan of the LEW/N rat, but, by contrast, a blunted response is observed only during the first 21 days after birth in F344/N rats and HSD rats. Thus, LEW/N rats appear not to emerge from the neonatal stress unresponsive period.

3. Genetic control of acute streptococcal cell wall (SCW) arthritis in LEW/N and F344/N rats:

   a. Breeding studies to understand the genetic control of acute SCW arthritis in LEW/N and F344/N rats have been completed. Attempts to correlate the arthritic phenotype to the previously described HPA axis defect are in progress, but data are still preliminary. The data obtained in the breeding studies indicate that arthritis severity in the F1 generation is intermediate between the 2 parental strains and that each parental phenotype reappears with frequencies of about 25% in the the F2 generation. These data indicate that a very limited number of

genes, and possibly a single polymorphic gene locus, regulates acute SCW arthritis severity. In light of these results, studies have been initiated to identify the chromosome(s) and ultimately the gene(s) controlling this phenotype. This objective requires the generation of a polymorphic genetic linkage map applicable to LEW/N and F344/N rats. In pursuit of this goal, to date we have:

i. Identified nearly 70 polymorphic markers distinguishing these two rat strains.

ii. Assigned nearly 50 of these polymorpic markers to specific chromosomes. Numerous regions of homology (synteny) with human and mouse chromosomes have been identified.

iii. Cosegregation of the arthritic phenotype and specific polymorphic genotypic markers has not yet been identified.

Significance to Biomedical Research and Program of the Institute

Peptidoglycan-containing bacterial cell wall fragments have received increasing attention in recent years as possible pathogenetic agents in some cases of chronic synovitis. This experimental animal model provides a powerful tool to explore the pathogenesis of chronic proliferative and erosive arthritis. We have defined parameters of both the host (cell wall distribution, inflammatory mechanisms, role of T-lymphocytes and various cytokines, neuroendocrine mechanisms, etc.) and the bacteria (type, resistance to biodegradation, fragment size) which influence the development of arthritis. Our recent studies on this model have provided an entirely new concept for the inherited basis of autoimmune/inflammatory diseases. Our data suggest that autoimmune/inflammatory diseases may result from defective activation of counterregulatory stress pathways involving the hypothalamic-pituitary-adrenal axis. Our more recent data suggest that we may be able to identify the genes that regulate these processes. These observations may have important implications for understanding autoimmune/inflammatory diseases in humans.

Proposed Course:

The role of the hypothalamic-pituitary axis in regulating disease susceptibility will continue to be a major focus of additional research. In particular, delineation of the molecular genetic basis for the defect in LEW rats will continue to be vigorously pursued. In addition, studies of cyclooxygenase, uteroglobin and CRH will continue.

Publications

1. Sternberg EM, Wilder RL, Chrousos GP, Gold PW. Stress responses and pathogenesis of arthritis. In Role of neuropeptides in stress pathogenesis in systemic disease. Editors R.G. Kaufmann, J.A. McCubbin, C.B. Nemeroff, Academic Press, Orlando, FL, 1991, pp. 287-300.

2. Sternberg EM, Wilder RL, Chrousos GP, Gold PW, Young WS III, Bernadini R, Calagero AE, Hill JM, Kamilaris T, Listwak SJ. The role of the hypothalamic-pituitary-adrenal axis in susceptibility to arthritis. In Proceedings of the international symposium on challenges of hypersecretion: ACTH, Cushings's syndrome and other hypercortisolemic states, ed. Ludecke DK, Chrousos G, Tolis G, Raven Prss, New York, 1990, pp. 183-188.

3. Sternberg, E.M., Wilder, R.L., Gold, P.W., and Chrousos, G.P. A defect in the central component of the immune system-hypothalamic-pituitary-adrenal axis feeedback loop is associated with susceptibility to arthritis and inflammatory disease. Annals of the New York Academy of Sciences 594: 289-292, 1990.

4. Wilder, R.L., Case, J.P., Crofford, L.J., Kumkumian, G.K., Lafyatis, R., Remmers, E.F., Sano, H., Sternberg, E.M., and Yocum, D.E. Endothelial cells and the pathogenesis of rheumatoid arthritis in humans and streptococcal cell wall arthritis in Lewis rats. Journal of Cellular Biochemistry 45: 162-166, 1991.

5. Karalis, K. Sano, H., Redwine, J., Listwak, S., Wilder, R.L., and Chrousos, G.P. A novel role for corticotropin releasing hormone: Local secretion and auto/paracrine proinflammatory actions in vivo. Science, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41066-09 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Synovial Tissues from Patients with RA and Related Conditions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	R. L. Wilder	Sr. Investigator	ARB/NIAMS
	H. Sano	Visiting Fellow	ARB/NIAMS
	L. Crofford	Medical Staff Fellow	ARB/NIAMS
	E. Remmers	Sr. Staff Fellow	ARB/NIAMS

COOPERATING UNITS *(if any)*

Holland Laboratories, American Red Cross
Developmental Endocrinology Branch, NICHD

LAB/BRANCH

Arthritis and Rheumatism Branch,

SECTION

Connective Tissue Disease Section

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The single feature most characteristic of rheumatoid arthritis is tumorlike proliferation of the synovium. During the past year, we have obtained additional evidence that platelet-derived growth factor, particularly a PDGF-B-like polypeptide, and heparin binding fibroblast growth factors play a role in regulating the growth and function of the connective tissue cells in diseased synovium.

We have also begun to study the role of neuropeptides in rheumatoid synovitis. Evidence has been obtained that locally produced corticotropin releasing hormone plays an important proinflammatory role in the disease process. These data provide additional evidence for interactions of the immune and central nervous systems in regulating inflammatory disease.

In addition, upregulated expression of other important inflammatory regulators such as cyclooxygenase and uteroglobin has been demonstrated.

All of these factors are expressed at low levels in synovium from osteroarthritic patients.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—862 878

Project Description:

Objectives:

The inner surface of the capsule of diarthrodial joints is lined by a delicate vascular connective tissue known as the synovial membrane. It consists of one or two layers of synovial lining cells (synoviocytes) overlaying an areolar or fibrous connective tissue. In conditions such as rheumatoid arthritis (RA), the surface layer of the membrane is markedly thickened and the deeper areolar tissue of the membrane is filled by infiltrating T lymphocytes, macrophages and plasma cells, producing a picture typical of chronic inflammation. Massive tumorlike proliferation of the synovial connective tissue cell populations are also characteristic. The objectives of this study are to characterize further this inflammatory tissue by study of tissue biopsy specimens and to delineate the molecular mechanisms underlying its pathological development.

Current objectives include:

1. In situ characterization of the cells and mediators in rheumatoid synovium. We are analyzing frozen and paraffin embedded sections of synovial tissues for expression of various markers using a variety of techniques such as immunohistochemistry. The tissues are also being examined for gene transcription. Particular emphasis, currently, is demonstrating the presence and sites of production of various growth factors, cytokines, neuropeptides, as well as important molecules in signal transduction.

2. In vitro culture studies. We are also characterizing the expression of a number of possible regulatory molecules such as cyclooxygenase, uteroglobin and various neuropeptides. Studies on cytokines such as platelet-derived growth factor and fibroblast growth factors are also being completed.

Major New Findings

1. PDGF and rheumatoid synovitis: Our previous studies have demonstrated the importance of PDGF-related polypeptides in rheumatoid synovitis. Followup studies have have demonstrated that specific receptors for PDGF, both types A and B, are markedly upregulated in rheumatoid synovial tissues in parallel with the upregulated expression of PDGF.

2. Cyclooxygenase expression in rheumatoid synovitis: Cyclooxygenase is the rate-limiting enzyme in prostaglandin synthesis. Prostaglandins are produced in abundance in rheumatoid tissues. Using specific antibodies and gene probes, we have demonstrated high level upregulated expression of cyclooxygenase in

rheumatoid tissues that correlates closely with the extent and intensity of mononuclear cell infiltration. Macrophages, fibroblastlike cells and endothelial cells express the enzyme at the highest levels. Low level and negligible expression was noted in osteoarthritic or normal synovium. These data parallel our studies in animal models.

3. Uteroglobin expression in rheumatoid synovitis: Uteroglobin is a potent phospholipase A2 inhibitor previously only described in the lung and uterus. We have demonstrated high levels of expression of this gene/protein in rheumatoid synovium. It is expressed at low levels in osteoarthritic synovium. These data also parallel our studies in animal models.

4. Neuropeptide expression in rheumatoid synovitis: Corticotropin releasing hormone (CRH) synthesis and release havebeen demonstrated in rheumatoid synovium. It is minimal in osteoarthritic synovium. Parallel studies, by us, in animals indicate that CRH is a potent local proinflammatory mediator, in addition to its role in regulating the hypothalamic-pituitary-adrenal axis. These data provide further insights into the links of the immune and central nervous systems.

Significance to Biomedical Research and Program of the Institute

An understanding of the primary mechanisms by which joint destruction is produced in diseases such as rheumatoid arthritis, requires knowledge about the types of cells in the inflammed tissues, their differentiation state, the mechanisms regulating their activity, etc. Our studies address these questions and provide insight into pathogenetic mechanisms. New therapeutic approaches are suggested from these studies.

Proposed Course

Studies are continuing with particular emphasis in defining the of cyclooxygenase, uteroglobin and various neuropeptides in rheumatoid synovium. An understanding of these mediators will undoubtedly provide insights into new therapeutic approaches.

Publications

1. Yocum DE, Wilder RL, Dougherty S, Gerber NL, Minor JR, Lesko LJ, Yarboro C, Berkebile CE, Klippel JH, Wahl SM. Immunomodulatory effects of cyclosporin A in rheumatoid arthritis. Arthritis Rheum. 33: 1310-1316, 1990.

2. Lafyatis R, Kim S-J, Angel P, Roberts AB, Sporn MB, Karin M, Wilder RL. Il-1 stimulates and all-trans-retinoic acid inhibits collagenase gene expression through its 5′ AP-1 binding site. Molec. Endocrinol. 4: 973-980, 1990.

3. Wilder, R.L. and Crofford, L.J. Streptococcal cell wall antigens and rheumatoid arthritis. In Rheumatoid arthritis, Editors J. Kalden, T. Maini, J. Smolen, Springer Verlag, in press.

4. Wilder, R.L. and Crofford, L.J. Do infectious agents cause rheumatoid arthritis. Clinical Orthopedics 265: 36-41, 1991.

5. Wilder, R.L., Case, J.P., Crofford, L.J., Kumkumian, G.K., Lafyatis, R., Remmers, E.F., Sano, H., Sternberg, E.M. and Yocum, D.E. Endothelial cells and the pathogenesis of rheumatoid arthritis in humans and streptococcal cell wall arthritis in Lewis rats. J. Cell. Biochem. 45: 162-166, 1991.

6. Remmers, E.F., Sano, H., Lafyatis, R., Case, J.P., Kumkumian, G.K., Hla, T., Maciag, T. and Wilder, R.L. Production of platelet-derived growth factor B chain (PDGF-B/c-sis) mRNA and immunoreactive PDGF B-like polypeptide by rheumatoid synovium: Co-expression with heparin-binding acidic fibroblast growth factor-1. J. Rheumatol. 18: 7-13, 1991.

7. Case, J.P., Lafyatis, R., Kumkumian, G.K., Remmers, E.F. and Wilder, R.L. Interleukin 1 regulation of transin/stromelysin transcription in rheuamtoid synoival fibroblasts appears to involve two antagonistic transduction pathways, an inhibitory, prostaglandin-dependent pathway mediated by cAMP, and a stimulatory, protein kinase C-dependent pathways. J. Immunol. 145: 3755-3761, 1990.

8. Remmers, E.F., Sano, H. and Wilder R.L. Platelet-derived growth factors and heparin-binding (fibroblast) growth factors in the synovial tissue pathology of rheumatoid arthritis. Sem. Arth. Rheum., in press.

9. Wilder, R.L., Remmers, E.F., Sano, H., Case, J.P., and Lafyatis, R. The cytokine network in rheumatoid arthritis. Brit. J. Rheumatol. 30 (suppl 2): 44-47, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41074-04 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)
Studies on etiology and pathogenesis of idiopathic inflammatory myopathy in humans

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Paul H. Plotz, M.D.	Sr. Investigator		ARB/NIAMS
	F.W. Miller, M.D., Ph.D.	CBER/FDA		CBER/FDA
	L. Love	FDA		FDA
	R. Leff	Sr. Staff Fellow		ARB/NIAMS
	D. Fraser	Clinical Associate	(formerly)	ARB/NIAMS
	S. Cochran	Clinical Associate		ARB/NIAMS
	N. Raben	Visiting Scientist		ARB/NIAMS
	R. Nichols	Staff Fellow		ARB/NIAMS
	R. Horwitz	IRTA Fellow		ARB/NIAMS

COOPERATING UNITS (if any)
none

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases Section

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Idiopathic inflammatory myopathy (polymyositis, dermatomyositis, and related disorders) is a family of inflammatory diseases in which disease-specific autoantibodies occur and for which there is considerable indirect evidence pointing to a viral etiology. We have over the past several years, seen and studied and collected serum, blood, and muscle specimens from well over 300 patients suspected of having myositis. We have collected epidemiologic information on many patients. We have completed an extremely sensitive search for candidate viruses by PCR in a subset of the specimens. We have extended studies of the diseases-specific autoantibodies to show that antibodies to tRNA often are present early in the course of disease. We have cloned, sequenced, and expressed histidyl-tRNA synthetase and are analyzing its structure and promoter, and are in the process of trying to clone other targeted synthetases. We have extended the analysis of HLA antigens in the sets of myositis patients defined by autoantibodies using the sequence specific oligonucleotide hybridization/PCR method.

Project Description:

Objectives:

1) By a study of a variety of parameters of disease, to uncover subsets of patients who may have a common etiology or pathogenesis.

2) To understand the relationship between the presence of a particular autoantibody and the disease in which it is found.

3) To seek direct evidence for candidate viruses in affected muscle of patients with myositis.

4) to study the structure, expression, and metabolism of target autoantigens in myositis.

Methods:

1) Clinical observation supplemented by epidemiologic data, analyzed by programs developed in part by our group.

2) Studies on autoantibodies by immunoprecipitation coupled with RNA labelling and sequencing.

3) PCR screening for viruses in muscle biopsies using a technique capable of detecting 1 to 20 molecules in a specimen.

4) Cloning, sequencing, and expressing aminoacyl-tRNA sunthetases using variations of a variety of standard molecular techniques.

Major Findings:

1) We have established a seasonal onset of disease in two sets of myositis patients whose category is defined by the presence of particular autoantibodies.

2) We have established that antibodies to the tRNA as well as to the synthetase are often present early in the course of myositis.

3) We have failed, with sensitive PCR, to find evidence for persistent nucleic acid of any of a number of candidate viruses in the biopsies of patients with myositis. Analysis of other viruses continues.

4) We have established the correct sequence of histidyl-tRNA synthetase, the principal target autoantigen so far identified in myositis (an incorrect sequence had been published) and have placed it in a suitable expression vector for the production of antigen for immunologic studies. We have also sequenced the upstream promoter and are in the process of analysing it.

Significance to Bio-Medical Research and the Program of the Institute

The recognition of subsets may clarify the etiology and pathogenesis of this disease family and shed light on a number of closely related rheumatologic diseases. Establishing that an autoantibody response is antigen-driven and related to disease activity enforces the view that specific etiologic agents should be sought.

Future Course:

We will expand our clinical, epidemiologic and serologic studies, especially focussing on defining HLA associations. Analysis of tissues for viral nucleic acid will be completed and pursued again when more evidence pointing to a particular agent is found. We will continue studies of autoantigen structure in order to understand how autoantibodies are driven in myositis, and what their relationship to the disease is.

Bibliography

Miller FW, Waite KA, Biswas T, Plotz PH. The role of an autoantigen, histidyl-tRNA synthetase, in the induction and maintenance of autoimmunity. Proc Natl. Acad Sci 1990;87:9933-7.

Love LA, Leff RL, Fraser DD, Targoff IN, Dalakas M, Plotz PH, Miller FW. A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine, 1991 (in press).

Leff RL, Burgess SH, Miller FW, Love LA, Targoff IN, Dalakas M, Joffe MM, Plotz PH. Distinct seasonal patterns in the onset of adult idiopathic inflammatory myopathy in patients with anti-Jo-1 and anti-signal recognition particle autoantibodies. Arthritis & Rheum (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT -

PROJECT NUMBER

Z01 AR 41075 03 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Picornavirus-induced chronic inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41076-04 ARB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Therapeutic trials in idiopathic inflammatory myopathies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Paul H. Plotz, M.D.	Sr. Investigator		ARB/NIAMS
F.W. Miller, M.D., Ph.D.	FDA		FDA
Richard Leff, M.D.	Sr. Staff Fellow		ARB/NIAMS
David Fraser, M.D.	Clinical Associate	(formerly)	ARB/NIAMS
Sarah Cochran, M.D.	Clinical Associate		ARB/NIAMS
M. Dalakas			NINDS
S. Leitman			CC Blood Bank
J. Hicks			CC Rehabilitation

COOPERATING UNITS *(if any)*

as above

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION: Connective Tissue Diseases Section

INSTITUTE AND LOCATION: NIAMS, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.65	1.65	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Severe idiopathic inflammatory myopathies (polymyositis, dermatomyositis, and related diseases) that respond poorly to corticosteroids are difficult to treat. We have undertaken three therapeutic trials for the treatment of such patients: a) a controlled double-blind trial of phasmapheresis and leukapheresis; b) a controlled, crossover trial of intravenous methotrexate with leucovorin rescue and a combination of methotrexate and azathioprine; c) an open trial of i.v. cyclophosphamide (completed).

The trial of apheresis has just been closed after 39 patients have completed it. Analysis of the results is underway. The trial of i.v. methotrexate and combination therapy continues, although patients with inclusion body myositis are no longer being entered because of poor results in the first 12 patients randomized.

Project Description

Objectives:

1. To determine whether plasmapheresis or lymphopheresis improves the course of severe polymyositis or dermatomyositis.

3. To determine whether methotrexate given intravenously or in combination with azathioprine improves the course of patients unresponsive to apheresis and/or conventional therapy.

Methods

a) Patients with PM or DM resistant to steroids or in whom severe steroid side-effects are present are randomized to 4 weeks (12 procedures) of phasmapheresis, lymphapheresis, or a sham procedure. Careful testing is done to determine the effect of therapy.

b) Patients who fail apheresis, are ineligible for apheresis, or who have inclusion body myositis are randomized to receive intravenous methotrexate or a combination of oral methotrexate and azathioprine. Those who have deteriorated at three months or who have failed to improve by six months are crossed over to the other therapy.

Major Findings:

1) Analysis is complete of the apheresis trial. No benefit found by any clinically significant measure for plasmapheresis or leukopheresis over a sham procedure. A manuscript will be submitted shortly for publication.

2) Patients with myositis continue to enter the iv methotrexate vs combination therapy trial.

Future Directions

Completion of the current trial. Planning for a trial of a promising bioengineered antibody is well underway.

Significance to Bio-Medical Research and the Program of the Institute

This is an attempt to improve the therapy of this very serious illness and to allow better study of its pathogenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41077-03 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Regulation of gene expression in normal and impaired fracture healing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41079-02 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of the Central Nervous System in Susceptibility to Arthritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Dr. Esther Sternberg

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Dr. Sternberg has moved to NIMH- The work cited under the above Project Number is now under: Z01 MH 02585-01 CNE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41080-03 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The use of MRI to detect inflammation in muscle of patients with myositis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Paul H. Plotz, M.D.	Sr. Investigator	ARB/NIAMS
	David Fraser, M.D.	Clinical Associate	ARB/NIAMS

COOPERATING UNITS *(if any)*

Dr. Joseph Frank	Radiology	Clinical Center

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases Section

INSTITUTE AND LOCATION
NIAMS, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.25	.25	

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- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [x] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In an attempt to provide an improved assessment of muscle inflammation in patients with myositis, we have evaluated MRI. In a group of myositis patients, muscle biopsy, laboratory tests, and the "STIR" image of the thighs were compared to an assessment of clinical disease activity. MRI was more sensitive than biopsy in detecting inflammation in these patients. The extent of muscle inflammatory changes could be assessed in patients since the entire muscle group is imaged. Magnetic resonance spectroscopy allows measurement of the metabolic state of muscle cells. Profound abnormalities of inorganic phosphate/creatine phosphate have been found in inclusion body myositis and in the tryptophan-induced eosinophilia-myalgia syndrome, and lesser abnormalities in polymyositis and dermatomyositis.

Project Description

Objectives:

To assess the extent of muscle inflammation in patients with inflammatory muscle disease.

Methods:

STIR (inversion recovery) images were performed on the thighs of patients with myositis. Biopsies, clinical charts, laboratory tests and MRI results were scored and compared by standard statistical methods. Magnetic resonance spectroscopy was performed on the thighs of patients with a variety of muscle diseases.

Major Findings:

MRS has demonstrated abnormalities in the relative levels of the important energy sources in muscle cells, creatine phosphate and ATP, in patients with polymyositis and dermatomyositis. This abnormality may account in part for the weakness experienced by patients, which is often way out of proportion to visible damage.

Significance to Bio-Medical Research and the Program of the Institute:

A method which can assess the extent of inflammation in myositis without involving a surgical procedure and which can be repeated would greatly assist planning therapy in inflammatory muscle disease.

The discovery of an abnormality of energy metabolism within muscle cells enforces the view that muscle cells are not simply destroyed, but pass through a phase where they are damaged and weak, but still functional. Understanding the nature of the damage may allow rescue of such cells before irreversible damage. This is especially important in a tissue such as muscle with very limited recuperative powers.

The STIR MRI image has been added to our routine workup since it provides useful information, directing muscle biopsy, or, on occasion, replacing it as a way of assessing the presence of inflammation.

Future Course:

MRI imaging has found a place in patient management, but its value, especially in assessing biopsy, will continue to be analyzed. MRS studies are not now being pursued, but we may return to them in the future.

Bibliography

Fraser DD, Frank JA, Dalakas M, Miller FW, Hicks JE, and Plotz PH. Magnetic imaging in the idiopathic inflammatory myopathies. J Rheumatol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41083-02 ARB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetics of Familial Mediterranean Fever

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*
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Ivona Aksentijevich, IRTA Fellow, Cellular Immunology Section, ARB/NIAMS
Luis Gruberg, Visiting Fellow, Cellular Immunology Section, ARB/NIAMS
Elon Pras, Visiting Associate, Cellular Immunology Section, ARB/NIAMS

COOPERATING UNITS *(if any)*
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Sheba Medical Center
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LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIAMS - Building 6, Room 112, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Familial Mediterranean Fever (FMF) is a rheumatic disease characterized by intermittent fever, serositis and/or synovitis; some patients also develop systemic amyloidosis. FMF is inherited as a single-gene, autosomal recessive disorder. The biochemical lesion of FMF, as well as the chromosomal location of the FMF gene, is unknown. The purpose of this project is to identify the FMF gene by the method of position dependent cloning ("reverse genetics").

During the first year of this project we prepared DNA samples from individuals in a panel of highly informative Israeli FMF families, and used RFLP analysis to exclude the FMF gene from 10% of the genome. Over the last year we have substantially extended this analysis. We have examined, and ruled out, a number of FMF "candidate genes," including serum amyloid A (SAA), interleukins 1, 6, and 8, dopamine beta hydroxylase, several lipocortin genes, and a number of genes encoding structural or regulatory proteins in the complement cascade. We have systematically examined another 100 DNA polymorphisms for possible linkage to the FMF susceptibility gene, and have thereby excluded the FMF gene from more than one third of the genome.

Moreover, we have identified a region on the long arm of chromosome 17 where one FMF susceptibility gene may reside, and we have evidence that there is at least one other FMF gene on another chromosome. By two point linkage analysis, the FMF gene mapped about 20 centimorgans telomeric to the DNA marker D17S74 (pCMM86), with a lod score of 2.48 (3.0 is required for definitive proof). We have subsequently identified four other markers in this area of chromosome 17 (D17S35, D17S40, D17S46, and growth hormone) which gave similar results. Multipoint linkage analysis, using D17S74 and D17S40 as fixed markers, demonstrated a maximum lod score of 3.28 approximately 10 cM telomeric to D17S40. We also found a significant probability of genetic locus heterogeneity in the families; only 16 of the 26 families examined showed evidence of linkage to chromosome 17. When this subset of families was considered independently in the multipoint analysis, the FMF susceptibility gene mapped to the 13 centimorgan interval between D17S74 and D17S40 (lod = 9.33).

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Objectives

The overall objective of this project is to find the gene which causes the human rheumatic disease Familial Mediterranean Fever. Because there are no biochemical clues to the genetic basis of this disease, we are using the method of position-dependent cloning ("reverse genetics"). Such a strategy has been used successfully to identify the genes encoding susceptibility to several human diseases, including cystic fibrosis and neurofibromatosis. This approach entails the use of genetic linkage studies within families of affected individuals to define progressively narrower intervals in which the disease gene must be found. The ultimate identification of the gene may involve the screening of likely candidate genes in the area of interest by a variety of approaches.

We have also identified a number of intermediate objectives. First, we have sought to establish a large panel of FMF families, and to obtain DNA samples from individuals in such families. This objective was largely accomplished during the first year of this project (cell lines were established from 347 individuals from 50 Israeli FMF families), although we have plans to continue accruing samples from both the U.S. and Israel over the next year. Second, we have attempted to establish the chromosomal location of the FMF gene through linkage analysis. During the last year we have mapped one FMF susceptibility gene to the long arm of chromosome 17, and we have further evidence that there is at least one other FMF gene. We will continue linkage studies in an effort to locate an additional gene or genes. Our third objective will be to establish a high resolution genetic map (i.e. 1 cM) and an even higher resolution physical map (i.e. 100 kb) for the relevant area(s) of the genome. Such a map, in conjunction with more family studies, will allow substantial narrowing of the area of interest. The final objective, which we would not anticipate approaching for at least the next year, is the actual identification of an FMF susceptibility gene.

Methods Employed

FMF patients have been identified at the Clinical Center of the National Institutes of Health, and at the Sheba Medical Center in Tel-Aviv, Israel. Peripheral blood lymphocytes were isolated from venous blood samples from these patients and selected family members, and cell lines were established using Epstein-Barr virus transformation. Genomic DNA has been isolated by standard methods; all cell lines are stored in liquid nitrogen for future use.

We have performed linkage studies using three different kinds of genetic polymorphisms. First, we have typed several erythrocyte antigens using standard serologic methods. Second, we have used Southern hybridization of radiolabeled DNA probes to genomic DNA immobilized on nylon membranes. Over 250 such probes, spanning the human genome, have been collected in our laboratory. Finally, we have used the polymerase chain reaction to amplify polymorphic dinucleotide repeats for analysis on sequencing gels.

Linkage analysis has been performed on a VAX workstation maintained in our laboratory, or on a VAX mainframe computer at the National Cancer Institute in Frederick, Maryland. Two-point linkage analysis was performed using the MLINK program; multipoint analysis was performed with LINKMAP. Genetic heterogeneity was tested using the HOMOG program.

Major Findings

The major findings of this project over the last year have been:

1. We have examined, and ruled out, a number of FMF candidate genes, including serum amyloid A (SAA), interleukins 1, 6, and 8, dopamine beta hydroxylase, lipocortins I, IIa-d, III, and V, and a number of genes encoding structural or regulatory proteins in the complement cascade.
2. We have have ruled out linkage between the FMF susceptibility gene and over 100 mapped polymorphic markers, thereby excluding the FMF gene from over one third of the genome.
3. We have established linkage between the FMF susceptibility gene and several markers clustered on the long arm of chromosome 17.
4. We have obtained evidence for genetic locus heterogeneity in FMF families, thus indicating that there is at least one other FMF susceptibility gene on another chromosome. There are no obvious phenotypic differences between individuals from families showing linkage to chromosome 17 and individuals from unlinked families.

Significance to Biomedical Research and Program of the Institute

Identification of the gene for FMF will provide insight into the genetic and biochemical mechanisms of rheumatic disease. This may allow for more rational design of pharmacologic agents for this and other rheumatic diseases. Successful characterization of this single-gene disorder will also establish technologies in the Intramural Program which may be applied to diseases with a more complex pattern of inheritance, such as lupus erythematosus and rheumatoid arthritis.

Proposed Course

During the next year we will pursue two avenues of investigation related to this project. First, we will perform studies to confirm and extend our observations regarding chromosome 17. To this end, we will first construct a more detailed genetic map covering the interval from D17S74 to D17S40. We will begin by studying markers which have been assigned to this general area of chromosome 17 by in situ hybridization or studies of somatic cell hybrids, but have not been placed on the linkage map. For these studies we will use DNA from the standard panel of families provided by the Centre d'Etude du Polymorphisme Humain, in collaboration with Dr. Michael Dean of the NCI. If we are unable to develop a genetic map of this interval with a 1 centimorgan resolution, we will screen members of a chromosome 17 specific library (available from American Type Culture Collection) to identify additional markers. Once we have established the requisite map, we will use our families (and additional families from Israel, if necessary) to place the FMF susceptibility gene relative to these markers. This step is an essential transition between the chromosomal mapping studies we have just completed and a serious physical mapping effort that we would anticipate during the fourth year of this project.

We will also continue genetic mapping studies, using Southern blots and PCR, to identify the chromosomal region(s) where one or more additional FMF susceptibility genes reside.

Publications

Sack, GH, Talbot, CC, McCarthy, BG, Harris, EL, Kastner, DL, Gruberg, L, and Pras, M. Exclusion of linkage between Familial Mediterranean Fever and the human serum amyloid A (SAA) gene cluster, Human Genetics, in press.

Gruberg, L, Aksentijevich, I, Balow, J, Dean, M, Pras, M, Kastner, DL Exclusion of candidate genes in Familial Mediterranean Fever, Cytogenet Cell Genet, in press.

Kastner, DL, Aksentijevich, I, Gruberg, L, Balow, J, Dean, M, Pras, M. Familial Mediterranean Fever: a 90 marker exclusion map and evidence for linkage to chromosome 17, Cytogenet Cell Genet, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT -

PROJECT NUMBER

Z01 AR 41088-01 ARB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neuroendocrine Factors in the Autoimmune Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	R.L. Wilder	Sr. Investigator	ARB/NIAMS
	J. Cash	Med. Staff Fellow	ARB/NIAMS
	L. Crofford	Med. Staff Fellow	ARB/NIAMS

COOPERATING UNITS *(if any)*

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Developmental Endocrinology Branch, NICHD

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent studies from animal models have rekindled interest in the role of the hypothalamic-pituitary-adrenal (HPA) axis and other neuroendocrine factors in rheumatoid arthritis (RA) and related autoimmune diseases. The pituitary-adrenal system was evaluated in 8 female patients with active RA treated with long-term low dose corticosteroids and were compared with 16 female age-matched controls. Data were obtained demonstrating elevated but submaximal ACTH and cortisol production in the patients with RA. The data are consistent with, but do not prove, the hypothesis that some rheumatoid arthritis patients may have inappropriately blunted HPA axis responses to inflammation.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864 939

Project Description: Z01 AR 41088-01 ARB

Background and Objectives

Recent animal model studies from our laboratories have suggested that blunted or inadequate hypothalamic-pituitary-adrenal (HPA) axis responses to inflammatory stimuli may predispose to the development of autoimmune, inflammatory diseases. The possibility that similar defects may exist in humans and predispose to chronic inflammatory disease has not been adequately addressed. Accordingly, our groups have initiated studies to characterize HPA axis and other neuroendocrine hormonal system responses in autoimmune inflammatory diseases.

Findings:

Ovine corticotropin-releasing hormone (CRH) stimulation tests were performed in 8 female patients with active rheumatoid arthritis treated with daily low dose prednisone and 16 age matched female controls. Patients were treated on the day of treatment, 12 hours after their last prednisone dose, and after withholding prednisone for 36 hours. Basal levels of plasma ACTH and to a lesser extent plasma cortisol levels were elevated prior to each test and significant increases in ACTH and cortisol were induced with CRH. The dose response relationship between ACTH and cortisol was shifted to the right suggesting that the therapy had led to mild adrenocortical atrophy compensated for by elevated ACTH concentrations. In other words, the patients exhibited elevated but submaximal ACTH and cortisol production. Submaximal production of these hormones may be inappropriate in the setting of chronic inflammation.

Significance to Biomedical Research and Program of the Institute

Our recent work in animal models has provided an entirely new concept for the inherited basis of autoimmune/inflammatory diseases. Specifically, we have proposed that defective activation of counterregulatory stress pathways involving the HPA axis may allow inflammation, once initiated, to proceed unchecked to produce chronic disease. The studies summarized here represent our initial attempts to extend these concepts to human autoimmune disease.

Proposed Course:

We plan to continue to expand our evaluation of HPA axis function in autoimmune diseases.

Publications:

1. Wilder, R.L. and Crofford, L.J. and Sternberg, E.M. Neuroendocrine aspects of autoimmunity. In Autoimmunity and Molecular Biology, Editor E. Brahn, Little, Brown and Co., Boston, in press.

ANNUAL REPORT

LABORATORY OF SKIN BIOLOGY

NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

October 1, 1990 to September 30, 1991

The Laboratory of Skin Biology is undergoing a rapid growth phase, and during the course of its first full year of existence, has successfully recruited a number of new scientists at several levels. The Laboratory presently consists of a section on molecular biology and structure. A newly appointed section on human genetics will join the Laboratory shortly. In addition, recruitment of clinical dermatologists to occupy a third integral section is underway.

At this time, the basic research of the Laboratory involves studies of the structure, function and gene expression of several of the major differentiation products and their accessory proteins expressed in normal epidermis, using biophysical, electron microscopic, biochemical, molecular biological and genetic approaches. The major emphasis of this work is directed toward an understanding of the possible role(s) of these proteins in keratinizing disorders of the epidermis.

Keratin Intermediate Filaments

Objectives

Terminally differentiating mammalian epidermal cells express large amounts of two intermediate filament (IF) chains, keratins 1 and 10, which are members of the class of IF proteins expressed in the cytoskeletons of virtually all eukaryote cells. These keratins, like all IF chains, have α-helical rod domains of precisely conserved secondary structures, but they have end domains enriched in glycines which presumably define the function of these filaments in epidermal cells. This work is directed toward an understanding of the structure and function of the keratin IF, their interactions with other components of the cytoskeleton and cell periphery, and role in keratinizing disorders of the epidermis.

Major Findings

1. Using limited proteolysis of disassembled forms of keratin IF, followed by electron microscopy, physico-chemical measurements, crosslinking and amino acid sequencing, neighboring coiled-coil molecules in the IF are aligned in alternating arrays of in-register and partly staggered antiparallel rows (Steinert).

2. By use of turbidity measurements of keratin IF assembly *in vitro*, IF are built from a rate-limiting nuclear particle which serves as a template for assembly. Furthermore, the data indicate that the dynamics of filament assembly *in vivo* and *in vitro* involve exchanges of multi-oligomeric units (Steinert).

3. Solid-state NMR studies on ^{2}H-lysine-labeled keratin IF reveals that the lysines are rather flexible, more so than leucines that line the hydrophobic core of the IF. This is consistent with and may explain the flexuous nature of keratin IF visualized *in vivo* and *in vitro* (Mack).

4. The end domains of keratins 1 and 10 contain glycine-rich sequences that are configured in the glycine loop motif. These keratins represent but two examples of many other structural and enzymatic proteins in biology that possess this motif. We postulate such sequences are important for the maintenance of the flexible and elastic recovery characteristics of the epidermis (Korge, Mack, Gan, Steven and Steinert).

5. The human keratin 10 chain is extraordinarily polymorphic with respect to the size and exact amino acid sequence of the glycine loop motif in the V2-subdomain only. The many different size alleles so far detected in the human population segregate in kindred families as normal Mendelian genetic traits. Such sequences containing trinucleotide polymorphic repeats are useful in genetic linkage analyses (Korge, Gan, McBride and Mischke).

6. Transgenic mice carrying the human keratin 10 gene have been constructed and will be useful in studies on the structure and function of the glycine loop motif (Korge and Compton).

7. A 38-residue peptide repeat is present on desmoplakin I, the bullous pemphigoid antigen and plectin, which shares major homology to the 1B portion of the rod domains of keratin chains, suggesting a possible mode of interaction between the keratin IF and cell junction and cytoskeletal crosslinking proteins (Steinert, Green and Stanley).

Profilaggrin

Objectives

Filaggrins specifically interact with keratin IF *in vitro* to form highly organized macrofibril structures. Likewise, it is thought the filaggrins are responsible for the proper alignment of the keratin IF *in vivo* during the terminal stages of epidermal differentiation and the formation of a flattened, dead cornified cell. The filaggrins are initially expressed in the form of a large polyprotein precursor, profilaggrin, which is subsequently proteolytically processed into individual functional filaggrin units by excision of a short hydrophobic linker sequence. The inappropriate expression of the profilaggrin gene system has been implicated in a number of

keratinizing disorders of human epidermis. Therefore, work in this area is directed to an understanding of the structure, function and expression of this gene system in normal and abnormally keratinizing epidermis.

Major Findings

1. Both mouse and human filaggrins consist largely of a repeating tetrapeptide β-turn motif containing a conserved distribution of charges. Synthetic peptides containing this motif but only the correct charge distribution can mimic the action of filaggrin in *in vitro* binding assays (Mack and Steinert).

2. Solid-state NMR experiments using ^{13}C-glycine-labeled or ^{2}H-lysine-labeled keratin IF reveal that filaggrin binds to the IF by way of ionic interactions with the rod domain, rather than end domains. This "ionic zipper" hypothesis is consistent with all of the available structural data (Mack).

3. Profilaggrin "minigenes" containing 3 or 0 repeats (instead of the normal 10-12 repeats) have been used for the production of transgenic mice. Preliminary data suggests these constructs are expressed appropriately in the mouse epidermis, but only at a very low level. New constructs containing additional flanking sequences are being used for additional experiments (Gan, Korge and Compton).

4. Many individual patients and their kindreds with certain keratinizing diseases have been collected for the purposes of performing genetic linkage analyses to assess the role of the profilaggrin gene in these diseases (Bale, DiGiovanna).

Loricrin and the Cell Envelope

Objectives

During terminal differentiation, epidermal cells deposit a layer of protein on the inner surface of the plasma membrane, termed the cell envelope, which contributes to the barrier function of the epidermis. The cell envelope becomes highly insoluble due to crosslinking by epidermal transglutaminases that form isodipeptide bonds, and by disulfide bonds. While several putative protein components of the cell envelope have been described, none seems to be a major component due to substantial differences in amino acid compositions and likely properties. However, we have recently described a new protein, loricrin, that fulfills the requirement for being the major structural protein component of the cell envelope of terminally differentiating epidermal cells and of perhaps other stratified squamous epithelia. We have initiated a major study of this system because very little is known about the structure and function of the cell envelope and its possible role in pathology.

Major Findings

1. We have isolated peptides from human epidermal cell envelopes that contain isodipeptide crosslinks and recognizable human loricrin sequences. This supports the notion that loricrin is the major cell envelope component, and is the first putative component shown to be directly crosslinked to the envelope (Steinert).

2. The human loricrin gene contains a single intron in 5'-untranslated regions and maps to the chromosomal position 1q21. This is the same location as the human profilaggrin gene as well as other genes for proteins expressed in the epidermis. Indeed, genetic linkage analyses reveal that the loricrin and profilaggrin genes are very tightly linked (Hohl, Yoneda and McBride).

3. By immunogold analysis, loricrin is initially deposited as composite keratohyalin granules in human epidermis, from which it is subsequently dispersed to the cell periphery (Cehrs and Steinert).

4. Using cell culture techniques, human loricrin gene expression is tightly controlled by both cell density and calcium concentration, and is inhibited by retinoic acid. The expression of the profilaggrin gene is essentially identical. Thus these two components are closely co-regulated during the terminal stages of epidermal differentiation (Hohl and Steinert).

Epidermal Transglutaminases

Objectives

The formation of the cornified cell envelope involves crosslinking of the protein components by isodipeptide N^{ε}-(γ-glutamyl)lysine bonds that are catalyzed by the action of a battery of several epidermal transglutaminases. At least three enzymes are now known to be expressed in the epidermis, the ubiquitous soluble C enzyme, the membrane-associated K enzyme, and the soluble glycine-rich pro-enzyme E enzyme. Comparatively little is known about these activities, their specificities and functions during terminal differentiation in the epidermis or even other tissues, and their possible roles in pathology.

Major Findings

1. A full-length clone for the human K enzyme has been characterized. It encodes a protein of about 89 kDa, has sequences that confer its membrane-binding properties, a conserved active site typical of the transglutaminase family, and has properties expected for the partially purified enzyme (Kim, Han, Idler, Chung and Steinert).

2. The gene for the human K enzyme has been characterized. It is about 15 kbp long, contains 15 exons and 14 introns. The structure of this gene suggests the

family of transglutaminase genes has arisen from a common ancestor which subsequently have been conserved (Kim).

3. The human K gene maps to chromosomal position 14q2-3 (McBride and Kim).

4. Partial sequences for the mouse E enzyme have been obtained by use of PCR analysis. The mRNA for this species is very rare and partially processed in the epidermis (Kim and Han).

5. Peptides corresponding to the amino- and carboxyl-terminal ends of human loricrin have been used as substrates for the three enzymes. The K_m value of the E enzyme is much lower than for the other enzymes, suggesting a preference *in vitro* and possibly *in vivo*, of the E enzyme for loricrin as substrate (Kim, Chung and Steinert).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41084-02 LSB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural features of keratin and related intermediate filaments

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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B. Korge, Sp. Vol., LSB/NIAMS; W. McBride, SR. Invest., LB/DCBDC/NCI
J. Mack, SSF, LSB/NIAMS; D. Parry, Professor, Massey Univ., NZ
Sp. Vol., Howard Univ; K. Green, Asst. Prof. Northwestern Univ.
J. Compton, Stf. Sci., Jackson Labs.; D. Mischke, Stf. Sci. Free Univ. Berlin
SSF, LSBR/NIAMS;
A. Steven, Chief, LSBR,NIAMS;

COOPERATING UNITS *(if any)*
LSBR/NIAMS; LMG/NICHD; DB/DCBDC/NCI; LB/DCMDC/NCI; Massey University, New Zealand; Northwestern University; Free University, Berlin

LAB/BRANCH
Laboratory of Skin Biology

SECTION

INSTITUTE AND LOCATION
NIAMS, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure, function and expression of the keratin intermediate filaments of human and mouse skin, and the related intermediate filament proteins of other cell types, are being investigated. These studies are designed to understand the structural features that determine how the rod domains of the chains pack to form the filament core. Current models are being tested using electron microscopic methods as well as by analysis of the products generated on limited proteolytic digestion of intact filaments or subfilamentous forms of them. The glycine-rich end domains of especially the keratin 1/10 filaments of epidermal cells are unique in biology. We believe these organize into a glycine-loop configuration. Current studies are designed to determine how these are packed and how they might interact with other macromolecules co-expressed in epidermal tissues. The glycine loop sequences on the human keratin 10 chain are extraoridnarily polymorphic in size and sequence. Using genomic clones to the human keratin chains 1 and 10, transgenic mice have been constructed to examine the expression characteristics of the genes as well as to probe in vivo the likely functions of the various portions of the chains, such as rod domain segments and glycine-rich end domains.

U.S. GOVERNMENT PRINTING OFFICE 1991 O—864 030

Project Description

Objectives

1. To determine the packing of the α-helical rod domain segments of the type I - type II keratin chains and type III vimentin chains of intermediate filaments.

2. To determine the likely secondary, tertiary and likely quaternary structures of a synthetic polypeptide corresponding to the 1A coiled-coil rod domain segment of vimentin intermediate filaments.

3. To determine the organization and molecular dynamics of the chains in keratin 1/keratin 10 intermediate filaments.

4. To determine the sequence, complexity, polymorphisms, secondary and possible higher-order structures of the glycine loops of the end domains of the keratin 1 and keratin 10 chains and other related intermediate filaments and unrelated structural proteins.

5. To study the expression, structure and function of keratin 1 and 10 genes in normal and abnormally keratinizing epidermis by use of the transgenic mouse model system.

6. To determine how the intermediate filament associated proteins, desmoplakin, its hemidesmosome equivalent (the bullous pemphigoid antigen) and plectin, interact with keratin intermediate filaments.

Methods

1. Scanning transmission electron microscopy is being utilized on vimentin intermediate filaments disassembled in low ionic strength buffers.

2. A 38-mer polypeptide corresponding to the 1A portion of vimentin has been synthesized and is being studied by sedimentation equilibrium, differential scanning calorimetry and solution state NMR methods.

3. Solid state NMR methods are being used to study the molecular dynamics of lysine-labeled and phenylalanine-labeled mouse epidermal keratin filaments.

4. Turbidometric and limited proteolytic methods are being used to study the organization of the keratin 1 and keratin 10 chains in intact or subfilamentous forms of mouse keratin intermediate filaments.

5. A survey of nucleic acid and protein sequence data bases is being used to search for proteins with the glycine loop motif.

6. Structural homology studies using available commercial or published methods as well as antibodies elicited against synthetic peptides are being used to analyze

the possible secondary and higher order structures of the glycine loop motif, and the structures shared by intermediate filament chains, desmoplakins, the bullous pemphigoid antigen and plectin.

7. PCR methods are being used with human DNA samples to study the sequence complexity and polymorphisms of the glycine loop sequences of the keratin 10 chain.

8. Genomic clones encoding the human keratin 1 and 10 chains are separately being used to prepare transgenic mice. The expression of the transgenes will be studied by use of indirect immunofluorescence, immunogold electron microscopy, *in situ* hybridization, and Western, Southern and Northern blotting techniques.

Major Findings

1. When examined by scanning transmission electron microscopy, unstained specimens of vimentin intermediate filaments disassembled into subfilamentous particles in 5 mM salt consist of a complex array of different-sized particles. Most are about 50 nm and have a mass of about 100 kDa and thus consist of a single coiled-coil molecule. Some other particles are about 65 nm long, and have masses of 200, 300 and perhaps 400 kDa, which indicates they contain 2, 3 or 4 molecules. Most of the particles are bent or chevron-shaped. These quantitative data conform to our earlier views on intermediate filament structure but do not support earlier claims in the literature that the smallest subfilamentous forms of these filaments are uniformly 50 nm long and contain 2 molecules aligned in register. Further work is in progress to generate more quantitative data and to examine slightly larger subfilamentous forms in an effort to understand the packing arrangments of neighboring molecules in intact filaments (Steven and Steinert).

2. In a complementary approach, native mouse keratin intermediate filaments containing keratins 1 and 10 have been partially disassembled at pH 2.6 and 9.8 and subjected to limited proteolysis. By use of electron microscopy with negative staining, biochemical experiments and amino acid sequencing, it has been possible to obtain data on the packing of neighboring molecules. The data support a unifying model in which the molecules are arranged in alternating rows of in-register and partly staggered antiparallel arrays. This model is consistent with the above scanning transmission electron microscopy data, as well as earlier models of filament structure based on predictions of ionic interactions. The model does not support several other ideas published in the literature (Steinert).

3. In a third approach, turbidity measurements have been done on solutions of keratin chains assembling into filaments *in vitro*. It appears that assembly is dependent on a rate-limiting step requiring the formation of either a hexamer and/or an octamer species. Physico-chemical, crosslinking, electron microscopy and amino acid sequencing experiments suggest the rate-limiting particle(s) consists of three or four molecules aligned antiparallel and partly staggered. Further filament assembly proceeds at a very rapid rate apparently involving the attachment of many different-sized species to this rate-limiting template. These data conform to the

above data, but it does not support several earlier models for filament structure published in the literature. A likely reason is that there have been a series of technical errors in these earlier experiments (Steinert).

4. Questions on the exact degree of overlap of neighboring molecules in intermediate filaments are being tested by crosslinking experiments. Crosslinked peptide species will be sequenced so as to elucidate the exact degree(s) of overlap of the intact chains. Such data will impose important constraints on any models for the higher orders of molecule alignment in intermediate filaments (Steinert).

5. Very recent experiments suggest that the epidermolysis bullosa simplex skin disorder may be due to mutations in the keratin 14 (and perhaps keratin 5) chains. An established method for correcting defects in structural proteins has been to use synthetic peptides. To explore whether peptide therapy is at least theoretically possible in this disease, a series of short synthetic peptides to various portions of the keratin chains are being synthesized and will be used in *in vitro* binding assays (Steinert).

6. A synthetic peptide corrresponding to the 1A rod domain segment of vimentin has been synthesized. In solutions of 1-1.5 X PBS it consists of a uniform dimer. It is now suitable for use in solution-state NMR experiments to solve its likely higher-order structures. This will be the first attempt to analyze in detail the structure of a coiled-coil. Such information will have important implications for future peptide therapy experiments (Mack).

7. We have found that the glycine-rich sequences on the end domains of the keratin 1 and 10 chains are configured as quasi-repeating peptides of the form $x(y)_n$, where x is usually an aromatic residue, y is usually glycine but occasionally another hydrophilic residue such as serine, cysteine, arginine, asparagine, and n can vary from 1-36 residues. Because the aromatic residues have a marked tendency to associate, we think the intervening glycine residues will be forced into a loop-like configuration, which we have termed the glycine loop. Sequences with a similar structural motif exist in loricrins, the major cell envelope protein also co-expressed with the keratins in terminally differentiating epidermal cells. Furthermore, searches in data bases indicate that this motif is common in several other classes of proteins, including: single stranded RNA and DNA binding proteins; plant cell wall proteins; plant hormones; certain calcium binding proteins; and possibly as the major structural protein of insect skins (resilin), first described in 1960. Our preliminary NMR data supports the view that the glycine loops will impart a highly flexible character to the structural protein and the entire macromolecular assembly or tissue. The data are consistent with the known physico-chemical properties of resilin (Steinert, Steven, Mack, Korge, Gan, Haynes).

8. Future experiments to understand the structure and function of the glycine-loop motif in proteins will proceed in several directions. First, resilin will be isolated from *Drosophila* for further characterization, and subsequently attempts will be made to determine its sequence from established cDNA libraries. Second, full-length cDNA clones of human keratin 10 and human loricrin chains are being assembled and will be configured in expression vectors for the expression of the proteins in the

presence of labeled amino acids to facilitate NMR studies. Subsequently, mutations will be introduced. Third, intact and mutated versions of the human keratin 10 chain will be used for micro-injection into cultured epithelial and epidermal cells to address structural-functional questions. The assay for such experiments will be immunofluorescence and immunogold electron microscopy with antibodies to the endogenous keratins and specific keratin 10 antibodies, and dynamics of phosphorylation at the known phosphorylatable sites and in the presence of phosphatase and kinase inhibitors (Korge, Mack, Steinert).

9. In order to study the molecular dynamics, mouse keratin intermediate filaments have been labeled *in vivo* and *in vitro* with ^{13}C-leucine or ^{2}H-lysine (which specifically label the rod domains) and ^{13}C-glycine and ^{2}H-phenylalanine (which specifically label the glycine-rich end domains). The latter two types of labeled filaments are useful to test the glycine loop hypothesis. The dynamics of the lysine- and leucine-labeled residues in the rod domains are quite different and provide new information about the flexibility of the rod domains. The data show that leucines are more constrained, presumably because they interact to form the hydrophobic core which stabilizes filament structure, while the lysines are more flexible in order to permit interactions with neighboring molecules in the filament. The same lysine-labeled filaments are also being used to test the ionic zipper hypothesis for the interaction of filaggrin with keratin filaments (Mack).

10. We have found that the size of the human keratin 10 chains is highly polymorphic in the human population. By use of PCR analysis of DNA samples from many different individuals, we have localized this polymorphism to the V2-subdomain containing the glycine loop motif. Variations by as much as 38 amino acid residues have been identified so far which involve deletions of several glycines or of whole glycine loops in the central region of the domain. These different size alleles segregate in kindred families by normal Mendelian genetic mechanisms, suggesting that the variations have been acquired over time during evolution and are not frequent transcription occurrences. Incidentally, this data provides an important trinucleotide repeat polymorphism which will be useful in future genetic linkage analyses of the type I keratin gene locus. Future experiments will examine the variations of the keratin 10 gene in pathological epidermis (Korge, Mischke, Gan and McBride).

11. Separate lines of transgenic mice bearing the human keratin 1 and keratin 10 transgenes have been prepared. Now that the basic constructs apparently contain all of the necessary information for correct temporal and tissue specific expression, mutations will be introduced in the keratin 10 construct in attempt to explore the structure and function of various portions of the rod domains, the phosphorylation sites, and the glycine loop hypothesis (Compton, Korge, Gan).

12. The proteins desmoplakin, a major protein component of desmosomes, the bullous pemphigoid antigen (apparently the hemidesmosome analog of desmoplakin) and the cytoskeletal associated protein plectin, all bind to keratin filaments and also share a region of sequence homology consisting of a 38-residue repeat. This peptide motif has a repeating charge distribution highly similar to the 1B rod domain segment of keratin (and other) intermediate filaments.

To test the hypothesis that this motif is directly or indirectly involved in binding of keratin filaments and thus for the structural organization of the filaments in epithelial cells, an antibody to a synthetic 57-mer peptide (containing 1.5 repeats) has been produced, and is being used for gel overlay binding assays, immunofluorescence and micro-injection experiments (Green, Stanley, Parry and Steinert).

Significance to Biomedical Research

A detailed understanding of the structure of keratin (and other) intermediate filaments is necessary to evaluate their functions in cells, their likely mode(s) of interaction with other cytoskeletal or cell peripheral components, and possible roles in keratinizing disorders of the skin and related epithelia. The use of transgenic systems will be useful in the development of mouse model systems for the study of certain disorders.

Publications

1. Steinert, P.M., Mack, J.W., Korge, B.P., Gan, S.-Q., Haynes, S. and Steven, A.C. (1991). Glycine loops in proteins: their occurrence in certain intermediate filament chains, loricrins and single-stranded RNA binding proteins. *Int. J. Biol. Macromol.* **13,** 130-139.

2. Steinert, P.M. and Freedberg, I.M. (1991). Molecular and cellular biology of keratin intermediate filaments. in *The Biochemistry and Physiology of the Skin,* ed. by Goldsmith, L.A., Oxford University Press, New York, pp. 113-147.

3. Tanaka, T., Parry, D.A.D., Klaus-Kovton, V., Steinert, P.M. and Stanley, J.R. (1991). Comparison of molecularly cloned bullous pemphigoid antigen to desmoplakin I confirms that they define a new family of cell adhesion junction plaque proteins. *J. Biol. Chem.* **266,** 12555-12559.

4. Rosenthal, D., Steinert, P.M., Chung, S., Huff, C.A., Johnson, J., Yuspa, S.H. and Roop, D.R. (1991). A human epidermal differentiation-specific keratin gene is regulated by calcium but not negative regulators of differentiation in transgenic mouse keratinocytes. *Cell Growth Different.* **2,** 107-113.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41085-02 LSB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Expression, structure and function of filaggrin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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4.7	4.5	0.2

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Filaggrin is a major differentiation product of terminally differentiating mammalian epidermal cells, that is thought to be involved in the aggregation and specific alignment of keratin intermediate filaments during the final stages of differentiation. Thus filaggrin is an important example of an intermediate filament-associated protein. We have isolated both cDNA and genomic clones which show that filaggrin is initially expressed as a large polyprotein precursor, filaggrin, that is subsequently proteolytically processed into individual functional filaggrin molecules. In the human system, the precursor consists of 3 allelic size variants, containing 10, 11 or 12 tandem repeats that segregated by normal Mendelian genetic mechanisms. In addition, these repeats show considerable sequence variation, so that any two repeats are only about 85% homologous to each other; so far, we find that of the 324 amino acid residues of the filaggrin repeats, about 40% of the positions are variable. We have constructed genomic fragments for the production of transgenic mice. We have begun a systematic analysis of regulatory sequences that control the expression of this gene system. We are studying the method of interaction of filaggrin with keratin intermediate filaments by use of solid state NMR techniques. Since there are a number of keratinizing disorders of the skin for which there is some evidence involving incorrect expression of the profilaggrin gene, we have begun a systematic search for the possible role of filaggrin in genetic diseases of keratinization.

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Project Description

Objectives

1. To determine the structure of the human profilaggrin gene

2. To determine the likely mechanism of interaction between filaggrin and keratin intermediate filaments.

3. To explore the expression of the human profilaggrin gene and mutations of it in normal human skin, in transfected cells in culture and in the transgenic mouse system.

4. To characterize the regulatory sequences and factors involved in the expression of the profilaggrin gene in normal and abnormally keratinizing epidermis.

5. To explore the role of filaggrin in various genodermatoses.

Methods

1. Genomic as well as cDNA clones are being isolated from several libraries for subcloning and sequencing.

2. Solid state NMR techniques are being used with unlabeled mouse filaggrin and keratin filaments labeled *in vivo* or *in vitro* in cell culture with ^{13}C-leucine, ^{13}C-glycine and ^{2}H-lysine.

3. Portions of the genomic clones have been joined to form minigenes containing 0 or 3 filaggrin repeats which are being used for the construction of transgenic mice.

4. Various portions of the genomic clones are being subcloned into CAT and other expression vector systems in attempts to identify potential positive and/or negative regulatory sequences.

5. Genetic linkage analyses as well as RFLPs and PCR analsyes of DNA samples from kindred families with normal epidermis as well as a variety of keratinizing disorders, is being done using standard techniques, in an effort to define the role of this gene system in disease.

Major Findings

1. Several cDNA clones containing one or more human filaggrin repeats, as well as two genomic clones encoding the 5'- and 3'-ends of the human profilaggrin gene have been isolated and partially sequenced. We have found significant sequence variations between adjacent repeats on the same clone. The accumulated data base suggests that at least 40% of the 324 amino acids/filaggrin repeat can vary.

In addition, RFLP analyses of several unrelated kindred families shows that there are at least 3 allelic size variants in the human population, containing 10, 11 or 12 filaggrin repeats, which segregate by normal Mendelian mechanisms (Gan and Steinert).

2. Detailed examination of the sequence variations show that they are conservative in nature. Secondary structure predictive methods indicate that such sequence variations do not significantly change the likely structure of human filaggrin. Likewise, mouse filaggrin has little overall sequence homology to human filaggrin, yet their secondary structures are essentially identical. Furthermore, available predictive algorithms suggest that up to 75% of the mouse and human filaggrin sequences consist of a quasi-repeating tetrapeptide β-turn sequence motif. Analysis of the sequences show a conserved pattern in which about 35% of the protein turns contain a positive charge, and about 10% possess a negative charge. To test this model and the hypothesis that this conserved charge motif is responsible for the binding by filaggrin of keratin filaments, a series of synthetic 20-mer peptides containing 5 such tetrapeptides was constructed, having net charges ranging from +5 to 0. The peptide of net charge +2 was about as effective in binding large numbers of filaments as filaggrin itself, using as criteria, both a weight-to-weight calculation, and also the size and complexity of the resultant macrofibrils as deduced by electron microscopy. Interestingly, both filaggrin and the +2 peptide peptide could bind large numbers of vimentin filaments as well (Mack, Gan and Steinert).

3. The possible mechanism of interaction of filaggrin with keratin (and other) intermediate filaments has been explored. Using unlabeled filaggrin and ^{13}C-glycine-labeled keratin filaments and solid-state NMR experiments, it is clear that there is little change in the molecular motions of the glycine-rich end domains of the keratin filaments in macrofibrils. This suggests that filaggrin does not bind filaments by way of their exposed end domains. However, when ^{2}H-lysine-labeled filaments are used (lysine preferentially labels the rod domains of the keratin filaments), the molecular dynamics of the lysine residues are considerably constrained in macrofibrils *versus* filaments alone. This seems to provide direct evidence that filaggrin binds to filaments by way of the rod domains through ionic and H-bonding interactions by an "ionic zipper" mechanism. This model can adequately explain how filaggrins may efficiently aggregate large numbers of keratin and vimentin filaments. We envisage that the conserved distributions of charged residues on filaggrins interact with the conserved periodic distribution of charges on the filaments. To date this is the first example of an intermediate filament - associated protein interaction that has been deduced (Mack).

4. Minigene constructs containing 0 and 3 filaggrin repeats of the human profilaggrin gene have been prepared and used for the generation of transgenic mice. One founder mouse and its progeny for each construct have been prepared. By indirect immunofluorescence, the epidermis of these mice express the human transgene specifically only in the upper layers of the epidermis and in the upper regions of the hair follicle canal. However, by Northern and Western blotting techniques, the level of expression of the human transgene was found to be low. This suggests that some, but apparently not all, of the regulatory sequences

required for normal and efficient expression of the human filaggrin gene were absent from these initial constructs. Therefore, new constructs have been made by insertion of an additional 9 kbp of upstream sequences which are now being used for the preparation of new transgenic mice (Gan, Compton and Korge).

5. Initial cloning and sequencing experiments could not unambiguously define the cap site of the human profilaggrin gene. Accordingly, RNAse protection and primer extension experiments by standard procedures with isolated foreskin RNA are in progress (Chipev and Markova).

6. Selected portions of the profilaggrin gene and surrounding upstream and downstream sequences have been subcloned in both orientations into pGEM, commercially available CAT vectors, and other vectors kindly provided by Drs Yuspa (LCCTP, DCE, NCI) and Schaffner (Switzerland). These are being transfected into cultured cells (HeLa, commercially available human epidermal keratinocytes and primary mouse epidermal keratinocytes) in an effort to define the various regulatory sequences that drive and control the expression of this gene system (Chipev and Markova).

7. Blood from patients and kindred family members with several keratinizing disorders (lamellar ichthyosis, ichthyosis vulgaris, epidermolytic hyperkeratosis and Darier's disease) have been collected, and are being transformed into permanent cell lines as a source of DNA for genetic linkage analyses. DNA is being characterized by Southern blotting. In initial studies, linkage analysis will be done to determine whether the disease phenotypes link to chromosome position 1q21, the known location of the human profilaggrin gene. If so, then PCR, pulse-field and sequencing experiments will be used in an effort to map which gene(s) in this region are involved and specifically whether the profilaggrin gene has been mutated. If not, then a systematic analysis of each of the chromosomes will be performed using known markers for each chromosome to look for other candidate genes (indirect genetics) (Bale, DiGiovanna, Gan and Compton).

Significance to Biomedical Research

Elucidation of the structure and expression of the profilaggrin gene system in both normal and abnormally keratinizing epidermis is crucial to an understanding of the function of the gene. Using our specific antibodies and genomic clones, we are able for the first time to initiate studies to define the precise role of this gene system in a number of major genodermatoses for which indirect data have existed for several decades.

Publications

1. Gan, S.-Q., McBride, O.W., Idler, W.W., Markova, N. and Steinert, P.M. (1990). The structure and polymorphisms of the human profilaggrin gene. *Biochemistry* **29**, 9432-9440.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41086-02 LSB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression, structure and function of loricrin, a major cell envelope protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cell envelope constitutes a thick layer of highly insoluble protein on the inside of the plasma membrane of cornified epidermal cells and of other stratified squamous epithelial cells. Of several putative protein components, none has hitherto proven to be a major component because of major differences in amino acid composition, levels of mRNA or of expressed protein. We have recently identified a new protein, termed loricrin, that fulfills all of the requirements of a major constituent of the cell envelope. Loricrin clones were first identified in a mouse epidermis, and now in more detail, from human epidermis. Loricrins are glycine-rich proteins that contain the highly flexible glycine loop motif. They are crosslinked in cell envelopes by isodipeptide $N^{epsilon}$-(gamma-glutamyl)lysine bonds. The possible role of loricrins in keratinizing disorders of epidermis is being investigated by transgenics, PCR, RFLP and genodermatoses analyses.

Project Description

Objectives

1. To characterize the crosslinking of loricrin to the cell envelope.

2. To characterize the expression of loricrin in the epidermis and in cultured keratinocytes.

3. To study the structural organization of the glycine loop motif of loricrin and its possible interaction with other keratinocyte cytoplasmic constituents.

4. To isolate and characterize the human loricrin gene and determine its chromosomal location.

5. To determine the function and expression characteristics of human loricrin in transgenic mice.

6. To determine the possible role of loricrin in keratinizing disorders of human epidermis.

Methods

1. Protein chemical methods are being used to isolate, purify and sequence peptides containing isodipeptide crosslinks from isolated cell envelopes.

2. Specific antibodies to mouse and human loricrins and filaggrins are being used for immunogold electron microscopy and immunofluorescence light microscopy to study the expression characteristics of loricrin in the epidermis and various other epithelial tissues.

3. Cell cultures of human keratinocytes are being used to study the expression of human loricrin and especially the role of effectors of epidermal differentiation such as Ca^{2+}, retinoids, etc on the expression of loricrin.

4. Secondary structure prediction and NMR methods are being used to study the possible structure and conformation of loricrins, especially with respect to their glycine loop structural motifs.

5. PCR methods are being used to explore the polymorphisms of the three glycine-loop domains of human loricrin in the population, and in different strains of mice.

6. Standard molecular biology techniques are being used to isolate and characterize genomic clones carrying the human loricrin gene, which are being assembled for the purpose of the production of transgenic mice.

7. Both skin and blood samples are being collected from patients and kindreds with keratinizing disorders of the epidermis for use in RFLP, PCR and genetic linkage analyses.

Major Findings

1. Human epidermis from newborn foreskins has been labeled in organ culture with ^{3}H-lysine, from which cornified cell envelopes were isolated and purified. Following digestion with proteinase K and separation in two dimensions by thin layer chromatography and electrophoresis, several labeled peptides were isolated and sequenced. Four peptides contained recognizable loricrin sequences as well as an isodipeptide crosslink formed by the action of the epidermal transglutaminases. This data has established that loricrin is (i) directly crosslinked to the epidermal cell envelope by such crosslinks; and (ii) supports our notion that loricrin is the major component of the cell envelope. Further studies are in progress to establish kinetic information about the deposition and subsequent crosslinking of loricrin and other putative protein components of the cell envelope (Steinert).

2. Specific antibodies to mouse filaggrin and mouse loricrin have been used for immunogold electron microscopy to study the deposition of these two proteins during terminal stages of epidermal differentiation. The filaggrin antibody labels large irregularly-shaped keratohyalin granules, termed F-granules. The loricrin antibody labels small round keratohyalin granules which we have termed L-granules. The two proteins have different ultimate fates in the epidermis, however. After dispersal from granules, loricrin appears throughout the cytoplasm of the "transition" cells and later becomes deposited on the intracellular side of the cell periphery at the cell envelope and persists throughout the stratum corneum layers. Filaggrin is dispersed throughout the cells among the keratin filaments, but the amount of filaggrin epitope diminishes rapidly above the "transition" layer and is essentially absent within 5 cell layers. Further work is planned to use anti-involucrin antibodies together with the above antibodies to obtain temporal information on the deposition of these various components during the final stages of epidermal differentiation (Steven and Steinert).

3. Similar experiments are now underway using antibodies and immunogold electron microscopy with human epidermis. In this tissue, the keratohyalin granules appear as "composite" granules in which the very small (0.2 μm) L-granules appear enmeshed with the larger irregular F-granules. Human filaggrin epitopes are distributed exactly as in mouse epidermis. Loricrin epitopes initially appear amongst the F-granules, rarely separately from them, and later exclusively appear along the cell envelope. In preliminary experiments using abnormally keratinizing human epidermal tissues, both filaggrin and loricrin epitopes are diminished or absent. Further work on tissue from a variety of disorders such as lamellar ichthyosis, ichthyosis vulgaris, epidermolytic hyperkeratosis and Darier's disease, is in progress in an effort to understand the expression and deposition characteristics of filaggrin and loricrin in the diseases (Steven, Cehrs and Steinert).

4. Both mouse and human loricrins contain three large domains of glycine-rich sequences, consisting of quasi-repeating sequences of the form $x(y)_n$, where x is usually an aromatic residue; y is usually glycine but may contain polar residues such as serine and cysteine; and n is 2-36 in the available loricrin sequences. This quasi-repeating motif has also been identified in the keratins and certain other proteins now known in biology. In the loricrins, the three domains are separated and interspersed by lysine and lysine + glutamine - rich sequences. We have postulated that the glycine-rich sequences form a novel structural motif known as the glycine loop. As for the other proteins, the loops have not been conserved in size or sequence between the mouse and human species. Secondary structural predictive algorithms suggest that the glycine loops are highly flexible and have little or no structural order, while the intermittent aromatic residues are markedly less flexible. In the case of human loricrin, the identification of four peptides crosslinked by the isodipeptide bond indicates that the loricrin molecule must be folded into a compact form. We envisage that this conformation thus permits interaction of the epidermal transglutaminases responsible for its crosslinking to the cell envelope, and the glycine loops project outward in a rosette-like manner where they may interact with the similar loops on keratin filaments (Steinert, Mack and Steven).

5. Using a specific 3'-noncoding probe, and rodent-human somatic cell hybrids and *in situ* hybridization, the human loricrin gene has been mapped to chromosomal location 1q21. Interestingly, this is the same general location as the profilaggrin gene as well as the involucrin gene, another putative protein component of the cell envelope. By linkage analyses, the profilaggrin and loricrin genes are very closely linked, probably within 1 mbp (McBride).

6. Two overlapping genomic clones encoding the human loricrin gene have been isolated and characterized. The gene simply contains a single intron of 1188 bp in the 5'-untranslated region. The available clones encompass about 10 kbp above the likely cap site and about 8 kbp below the polyadenylation signal sequence (Hohl, Idler, Yoneda).

7. These clones have been reconfigured in a form suitable for the construction of transgenics. Because our available mouse and human antibodies cross-react, the genomic clones have been modified by insertion of the substance P amino acid sequence in frame immediately before the termination codon of the human loricrin. Specific antibodies to the substance P epitope are commercially available. The resulting construct of 21 kbp has been assembled into a pGEM vector and has been used for the injection of fertilized mouse eggs for the production of transgemic mice, which will be characterized following breeding of F1 progeny. Once the appropriate expression characteristics of this basic construct have been established, a variety of mutations will be introduced in glycine loop and likely transglutaminsase substrate domains to study the expression, structure and function of human loricrin (Yoneda).

8. Preliminary PCR data suggests that the sizes of the three glycine loop domains in human loricrin vary in the human population. In this regard, they may be analagous to the allelic polymorphism encountered in similar sequences of the human keratin 10 gene. The extent of this possible polymorphism in the human

loricrin gene will be fully explored. Similarly, the available mouse and human loricrin sequences show wide variations. Therefore, different strains of mice will also be studied (Yoneda).

9. The expression characteristics of human loricrin in cultured keratinocytes has been explored. By use of both Northern and Western blots, loricrin is expressed optimally only at a very specific level of Ca^{2+} and near confluence of cell growth. Its expression is inhibited by retinoids. Since the same conditions are also optimal for expression of the profilaggrin gene, it seems that the expression of these two late differentiation products is closely coordinated, so that similar regulatory sequences and *cis* -acting elements may control both. Accordingly, the available 10 kbp of upstream sequences of human loricrin gene are in the process of being sequenced, to facilitate future studies on the regulation of its expression (Hohl and Yoneda).

10. Blood samples from patients and kindreds with a variety of keratinizing disorders (see item 3 above) are being collected for the purpose of initiating genetic linkage analyses on the potential role of the loricrin gene in keratinizing disorders (Bale and DiGiovanna).

Significance to Biomedical Research

Determining the structure and expression of the human loricrin gene system is of vital importance in understanding the role of loricrin in normal epidermal differentiation and its potential role in keratinizing disorders of the epidermis.

Publications

1. Steven, A.C., Bisher, M.E., Roop, D.R. and Steinert, P.M. (1990). Biosynthetic pathways of filaggrin and loricrin - two major proteins expressed by terminally differentiated epidermal keratinocytes. *J. Struct. Biol.* **104,** 150-162.

2. Hohl, D., Mehrel, T., Turner, M., Lichti, U. Roop, D.R. and Steinert, P.M. (1991). The characterization of human loricrin: structure and function of a new class of epidermal cell envelope proteins. *J. Biol. Chem.* **266,** 6626-6636.

3. Hohl, D., Lichti, U., Breitkreutz, D., Steinert, P.M. and Roop, D.R. (1991). The transcription of loricrin in vitro is induced by calcium and density and suppressed by retinoic acid. *J. Invest. Dermatol.* **96,** 414-418.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 AR 41087-02 LSB

OD COVERED
October 1, to September 30, 1991

OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Epidermal transglutaminases

CIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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K APPROPRIATE BOX(ES)
(a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

MARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transglutaminases form isodipeptide crosslinks between acceptor amide groups of glutaminyl residues and donor epsilon-NH_2 groups of lysines. In the epidermis, and other stratified squamous epithelial tissues, these enzymes are thought to be involved in the crosslinking of putative protein components to form the insoluble cell envelope. Using molecular biology approaches, we have found that there are 3 different transglutaminase activities in normal human and mouse epidermis. These are known as the K, C and E enzymes. Work is in progress to isolate and characterize cDNA and genomic clones encoding each of these. The aim of these studies is to determine the likely functions of these different activities in normal and abnormally keratinizing epidermis.

Project Description

Objectives

1. To isolate and characterize cDNA clones encoding the three different transglutaminases expressed in the epidermis and to determine their likely protein structures.

2. To localize these activities in epidermal and related epithelial tissues and to explore their expression characteristics in normal and abnormally keratinizing human epidermis.

3. To determine their genomic structures and chromosomal locations.

4. To characterize the regulatory sequences and factors involved in the expression of the transglutaminases K and E in normal and abnormally keratinizing human epidermis.

5. To determine the likely role and substrate specificities in cell envelope formation in normal and abnormally keratinizing human epidermis.

Methods

1. Synthetic oligonucleotides and PCR methods will be used to isolate both cDNA and genomic clones from appropriate cell culture, foreskin and genomic libraries, using standard molecular biology techniques.

2. Indirect immunofluorescence, *in situ* hybridization, immunogold electron microscopy, Northern, Southern and Western blotting techniques are used to localize the expression of the transglutaminase enzymes.

3. *In vitro* assays of synthetic peptides derived from loricrins and involucrin are being used to assess the related activities of the enzymes.

Major Findings

1. Full-length cDNA clones for the human transglutaminase K, expressed in intact epidermis as well as cultured epidermal keratinocytes, have been isolated and characterized (Kim).

2. By Northern blotting techniques, we have found that the same or very similar transglutaminase K activity is expressed in a variety of other non-epithelial tissues, including B16 murine melanoma. Interestingly, in the latter case, the activity is extracellular, rather than intracellular as in epithelial tissues. Experiments are underway to clone and sequence the melanoma enzyme to determine whether it is the same gene product and modified post-transcriptionally or post-synthetically, or

whether it is expressed from another gene. Related experiments using Southern blot assays, however, suggest that the transglutaminase K may be a single-copy gene/haploid genome in human (Chung, Han, Idler and Kim).

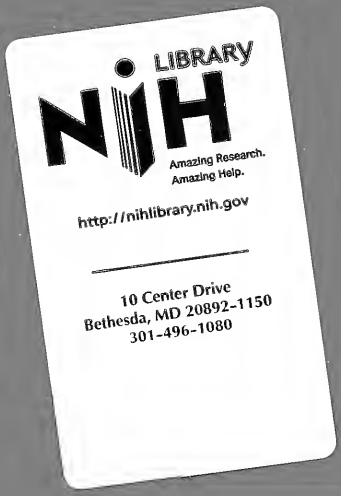
3. By use of both human-rodent somatic cell hybrids and *in situ* hybridization with biotin-labeled probes, the human transglutaminase K gene maps to the chromosomal position 14q2-3 (Kim and McBride).

4. The human transglutaminase K gene has been isolated and characterized. It is about 15 kbp in length and contains 15 exons interspersed by 14 introns. The splice positions of these introns have been precisely conserved in comparison to the two other transglutaminase-like enzymes so far characterized (factor XIIIa and the so-called band 4.2 protein). Interestingly, the K gene is by far the smallest of these: the other two are estimated to be >200 kbp and >65 kbp, respectively. The human transglutaminase K gene clones so far characterized contain only about 2 kbp of upstream sequences and 5 kbp of downstream sequences. Other clones containing flanking sequences will be characterized. Such sequences will be necessary to identify and characterize the regulatory sequences of this gene (Kim).

5. A specific antibody elicited to the unique carboxyl-terminus of the human transglutaminase K has been produced. This will now be used for expression studies (Steinert).

6. Using PCR methods with oligonucleotide primers designed from amino acid sequences provided by Gorman, Kim and Han have isolated cDNA clones encoding the mouse transglutaminase E. To date, about 700 bp of an estimated 3.3 kbp of mRNA sequences have been defined. Northern blotting analyses have shown that the E mRNA species is very rare; it is expressed only in intact epidermal tissues, and not by cultured keratinocytes. Indeed, there are no clones for coding portions in a human foreskin cDNA library. Furthermore, it appears that the 5'- and 3'-ends of this mRNA are partially processed in epidermal tissues, as for other late differentiation products of the epidermis such as profilaggrin and loricrin. In continuing experiments, PCR amplification of first-strand cDNAs modified by adaptor primers together with primers from existing specific sequence information, are being used. In this way, it is hoped that full-length sequence information can be obtained by "walking" up and down the rare and degraded mRNA. As soon as more specific mouse transglutaminase E cDNA sequence information becomes available, these experiments will be repeated to generate sequence data for the human E cDNA. Preliminary protein chemical data indicates that the mouse and human E enzymes have significantly different properties, especially with respect to their net charges and glycine contents. Marked differences between these two species have been found for other epidermal products such as profilaggrin, loricrin and the keratins. In addition, specific sequence data will be used to isolate genomic clones for the human transglutaminase E (Kim, Han and Steinert).

7. Synthetic peptides corresponding to the amino- and carboxyl-terminal ends of human loricrin have been synthesized. These contain known sites of isodipeptide bonds. These have been used as substrates for the three known transglutaminse enzymes expressed in the epidermis. Using an *in vitro* assay, kinetic data reveal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